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STUDY OF SILICA NANOPARTICLE COMPOSITE ON SILICA-HYDROGEN PEROXIDE COMPLEXATIONS AND THEIR EFFECTS IN CATECHOL BASED ADHESIVES

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STUDY OF SILICA NANOPARTICLE COMPOSITE ON SILICA-HYDROGEN
PEROXIDE COMPLEXATIONS AND THEIR EFFECTS IN CATECHOL BASED
ADHESIVES

By

Rattapol Pinnaratip

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Biomedical Engineering

MICHIGAN TECHNOLOGICAL UNIVERSITY

2020

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This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Biomedical Engineering.

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Dedicated to my loving mother

Sureerat Pinnaratip

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Preface

The material used in Chapter 1 of this dissertation was previously published in *Advanced healthcare materials*, 8(11), p.1801568. This article was republished in parts which are relevant with permission from ACS Publications. The material used in Chapter 2 of this dissertation was previously published in *Biomedical Materials*, 13(2), p.025003. This article was reproduced in parts which are relevant with permission from IOP Publishing, LTD. The material used in Chapter 3 of this dissertation was previously published in *ACS Biomater. Sci. Eng.*, 2020, (page not yet assigned). This article was reproduced in its entirety with permission from ACS Publications.

In Chapter 3, I have synthesized all materials, collected, and analyzed the data, as well as written the manuscripts for first-authored publications that are a part of this dissertation. Dr. Bruce P. Lee has guided me through my research and also supported my work with his grants. Dr. Rupak M. Rajachar has guided me through the biological aspect of the research. Applied Chemical and Morphological Analysis Laboratory (ACMAL) at Michigan Technological University's facility and staff have given me support for all the FE-SEM, SEM, TEM, and XRD. Meijia Li performed the Brunauer, Emmett, and Teller (BET) surface area analysis with the supporting grant from Dr. Yun Hang Hu. Pegah Kord Forooshani has helped as a supporter for the cell experiments.

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The abstract, latter part of Chapter 1 (page 8-page 16), Chapter 2, summary, and future outlook sections were specifically written exclusively for this dissertation.

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After I got my scholarship from the Thai Government Scholarship program per request of Chiang Mai University, Chiang Mai, Thailand. I got accepted to Michigan Technological University as a Master student, following my undergraduate's project advisor, Dr. Bovornlak Oonkanond, who is a Ph.D. graduate from Tech in the class of 2003. I started my Master's student's journey in Dr. Rajachar's Lab back in the spring of 2013, and then I later join Dr. Bruce P. Lee's lab in early Fall 2013. My background was in Biomedical Engineering with a focus on bioinstrumentation and electronics with minimal knowledge of cell biology and material science. I still remember the first time that I discuss the silica particle synthesis project with Dr. Rajachar and found out that my knowledge in that particular field is still lagging. Just after a six-month trial period, Dr. Rajachar introduced me to Dr. Lee, I still sometimes wonder what Dr. Lee saw in me that made him accept me into his lab group as my co-advisor. However, what I am very confident about is that from that day onward until today, both of my co-advisors have been constant supports to me in achieving all my academic goals. Dr. Rajachar was strict and kept pushing me forward in the first few years as he believed in me. This made me reached a new height in academic thinking and research conduct. Dr. Lee has always guided me in the right direction, and his advice has proved to be of utmost value in my pursuit to learn and grow as an independent scientist. Both kept reminding me that I will have to make things simpler and explain my finding in the easier to understand manner; I still keep Dr. Rajachar's comment with me (a single "Simple" on a piece of paper). I would always like

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Abstract

Mussel adhesive proteins contain catechol moiety, which allows the protein to crosslinked, solidify, and adhere to surrounding surfaces even under wet conditions. Incorporating the catechol moiety into polymeric adhesive resulted in bioadhesive, which still functions under wet conditions. However, the adhesive must be oxidized in order to crosslink and adhere to surfaces. The oxidation process of catechol adhesive was proven to produce singlet oxygen, superoxide, and hydrogen peroxide (H_2O_2). These reactive oxygen species, with lack of control, can wreak havoc to the biological system causing poor healing and undesired biological response.

Here, we studied the silica particle system as a means to control H_2O_2 concentration. After an exploratory investigation on silica particle synthesis and modification, acid-treated silica particle (AHSi) was developed with a highly hydrophilic surface. We then explore the use of reinforcement phase incorporation, creating particle-adhesive composites and studied the effect which the particle incorporation imparts into the adhesives. The model adhesive is polyethylene glycol functionalized with glutaric acid and dopamine, creating a biodegradable adhesive hydrogel. We demonstrated that incorporation of a model particle, silica particle, into a catechol adhesive resulting in a mechanically stronger adhesive with an increase in stiffness, adhesion strength, and structural integrity even after partially degraded. Moreover, the composite adhesive was gelled faster and degrade slower than native PEG-DA adhesive. The composite also

demonstrated a reduction in the concentration of H_2O_2 . The particle not only reduces H_2O_2 but also found to be releasing soluble silica in a biologically relevant concentration further improve their bioreactivity. The silica nanoparticle incorporated catechol-based composite demonstrated a reduction in cytotoxicity on rat dermal fibroblast, human keratinocyte, and human tenocyte, three types of cells that react differently to elevated oxidative stress.

Interestingly, all cell types have demonstrated an increase in cell proliferation, raising the possibility of developing the composite adhesive further. The last part of the study involved a prediction model that helps narrow down the formulation to be tested in vivo. Full-thickness dermal wound model in mice was utilized to study the predicted formulation. The results from the animal model suggested that PEG adhesive alone can alter the biological response with accelerated wound healing. However, the incorporation of AHSi proved to successfully bridge the gap between accelerated wound healing and better wound remodeling. This dissertation describes various strategies used to tune the H_2O_2 concentration released from catechol adhesives to tune its biological response which involved silica particle modification with minimal change in chemical composition, and the selection of adhesive formulation to enhance the wound healing and wound remodeling.

1 Multifunctional Catechol Adhesive Composite Design¹

1.1 Introduction

Wound closure, surgical repair, and reconstruction of traumatized or degenerated soft tissues frequently occur in medicine. These procedures involve rejoining biological tissue surfaces or securing implantable devices (e.g., surgical meshes, grafts, etc.) to living tissues¹⁻³. Conventionally, the use of sutures and staples have been the primary means to achieve such wound closure or implant fixation⁴⁻⁶. However, these methods lead to additional trauma, leakage, and a lack of tissue integration resulting from significant compliance mismatch between the tissue and the fixation device⁷⁻¹⁰. Biomedical adhesives can potentially overcome these limitations and provide additional benefits such as reducing surgical time, minimizing treatment invasiveness, reducing additional trauma, and preventing body fluid leakage^{1-3, 11, 12}.

Despite the potential benefits of tissue adhesives, commercially available bioadhesives have limited applications due to limited functionality. Specifically, existing adhesives are primarily designed to have one key function, which is to provide mechanical support for the repaired tissue. To expand the potential utilization of bioadhesive in medicine, researchers have reported novel multifunctional bioadhesives with improved performance. Multifunctional bioadhesives not only need to meet the requirement of

¹ Parts of the material contain in this chapter was previously published in *Advanced healthcare materials*, 8(11), p.1801568. <https://onlinelibrary.wiley.com/doi/full/10.1002/adhm.201801568>

adhering to biological tissues but also have added functions aimed at improving the outcome of the wound healing process. Biomedical adhesives can be engineered to have an antimicrobial property to minimize microbial growth and prevent acute complications. Some bioadhesives are also designed to self-heal when damaged, which will prolong the service life of the adhesive. Stimuli-responsive bioadhesives are designed to respond to externally applied triggers such as pH, electricity, temperature, light, or changes in the wound environment to promote cellular migration, tissue-specific gene expression, and native tissue healing. The ability to respond to externally applied stimuli enables these adhesive to release therapeutic drugs or change their adhesive property on command in response to dynamic physiological conditions.

Recent advances in mono-functional bioadhesives have been reviewed elsewhere^{1-3, 13, 14}, and will not be the focus of this review. Instead, this review paper focuses on multifunctional bioadhesives, which we categorize into either passive or active by design (**Figure 1**). Passive multifunctional bioadhesives contain inherent compositions and structural designs that can carry out additional functions (e.g., antimicrobial property, self-healing ability, etc.) without the need for added external factors or influences. Conversely, active multifunctional bioadhesives respond to environmental changes (e.g., pH, electricity, temperature, light, biomolecule concentration, etc.), which triggers a chemical or structural change within the adhesive that initiate the added function of the adhesive. These environmental triggers could be applied externally by the user after the adhesive has been deployed or internally triggered resulting from the natural physiological responses to the presence of the adhesive (e.g., normal wound healing

response) or physiological conditions of the biological environment (e.g., elevated insulin concentration in a diabetic patient). In this review, we address fundamental criteria for designing a bioadhesive, review the existing bioadhesives in the market, and review passive and active multifunctional bioadhesives reported in the literature.

Figure 1. Multifunctional biomedical adhesive design considerations. The fundamental attributes of any successful biomedical adhesive include an application-specific balance of appropriate cohesive and adhesive interactions coupled with the biocompatibility of the bulk adhesive material as well as any products generated during the curing process or degradation of the adhesive with time. Passive and active design elements can be used to ultimately tailor or refine these core attributes in multi-functional bioadhesives to more specifically target an overall desired outcome.

1.2 Key criteria for biomedical adhesive design

Regardless of the type, function, or the intended application of a bioadhesive, there are three key criteria that all bioadhesives should satisfy ^{1-3, 11-14}. These criteria include the ability to form strong interfacial bonds, the ability to retain strong bulk cohesive properties after curing, and the necessity to remain biocompatible throughout the lifetime of the bioadhesive. This section reviews these key design criteria for bioadhesives.

1.2.1 Interfacial interactions

Biomedical adhesives need to form strong interfacial bonds with tissue substrates ^{11, 12}. For fixation of surgical implants, bioadhesive may need to adhere strongly to surfaces with different surface properties and chemical makeup (i.e., metal, polymer, ceramic). These interfacial bonds can include chemical crosslinking, physisorption, diffusion, electrostatic interaction, physical interlocking, and micromechanical interactions. Most bioadhesives utilize more than one type of interaction to bond to these surfaces. Chemical crosslinking consists of strong interfacial covalent bonds (**Figure 2**). Commonly used interfacial chemistries involve activated ester (e.g., *N*-hydroxysuccinimide (NHS) activated acid group), isocyanate, and aldehyde functional groups, which are reactive toward primary amine found on the surface of soft tissues (e.g., -NH₂ of lysine) ¹³. Catechol mimics the reactivity of the adhesive amino acid, 3,4-dihydroxyphenylalanine (DOPA), found in mussel adhesive proteins ^{15, 16}. The catechol-modified adhesive is capable of binding to both tissue and inorganic substrates (i.e., polymer, metal).

Additionally, catechol-dimerization has also been shown to enhance wet adhesion toughness¹⁷. Physisorption involves weak intermolecular forces such as van der Waals interaction resulting from positively and negatively charged regions within bonding molecules¹⁸. Physisorption is known to perform poorly within a moisture-rich environment as water interferes with the weak molecular interaction¹⁹. As such, a bioadhesive that relies solely on van der Waals interaction may not be effective in a biological system. Diffusion of polymer networks across interfaces occurs when the adherent and substrate surfaces are mutually soluble, and their polymer chains have sufficient mobility¹⁸. Polymer diffusion is favorable in highly hydrated conditions and may be limited to bioadhesives designed for moisture-rich environments, such as blood vessel or digestive tracks. Ionic adhesives with positive or negative charges can bind to surfaces with the opposite charge through electrostatic interactions²⁰. Electrostatic interaction can be modulated by changing the ionic environment surrounding the adhesive, which may be useful in designing smart adhesives that respond to changes in pH.

Depending on the desired application, there may be different performance criteria that the bioadhesive needs to satisfy. For example, Pascual et al.²¹ reported that abdominal wall repair using a surgical mesh required a fixation strength of 55-60 N, and cyanoacrylate-based adhesive was equally as successful as suture fixation. Sutures have high tensile strength (8 to 15 N)²² and require minimal footprints for repair. However, the performance of bioadhesives is highly dependent on the overlapped surface area between the adhesive and the substrates. To perform microneural anastomosis, Choi et al.²³

overlapped the epineuriums of the two nerve ends and then applied cyanoacrylate-based adhesive. This method was comparable to sutured anastomosis. To function as a lung sealant, a burst pressure of 30-60 mmHg may be sufficient^{3, 24}. However, a significantly higher burst pressure (200-400 mmHg) may be required for sealing renal veins²⁵ or sutured intestinal anastomosis²⁶. Hence, to design an effective adhesive, it is necessary to understand the parameters specific to the surgical procedures.

Figure 2. Interfacial crosslinking chemistries between adhesive functionalized using NHS-activated ester (**A**), isocyanate (**B**), aldehyde (**C**), and catechol (**D**) with nucleophilic functional groups (e.g., -NH_2 of lysine) found on soft tissues. Catechol can also form coordination bonds with metal oxide surfaces, which may be present on the surface of an implant or medical device (**E**).

1.2.2 Curing of adhesive and cohesive properties

In addition to forming strong interfacial bonds, a bioadhesive must also develop cohesive strength after it has been applied. The adhesive must remain stable for the duration of its service life to provide adequate support for a wound to heal. For an injectable bioadhesive, the adhesive must be able to transform from the liquid state into the solid-state under mild physiological conditions. This physical change should be rapid (typically 3 to 5 minutes) to minimize surgical procedure time¹. The curing process typically involves the formation of intermolecular crosslinking, which can be achieved via the mixing of reactive components or activated by external stimuli such as ultraviolet (UV) light²⁷. However, the use of longwave UV (e.g., UVA) is preferred over medium-wave UV (UVB) to minimize tissue damage²⁸. After curing, the bioadhesive must possess the bulk mechanical strength necessary to withstand mechanical forces applied by the surrounding tissues. A tissue adhesive with adhesive strength and biomechanical properties matching those of native tissues can potentially be used to repair tissues that routinely experience repeated loads (e.g., cartilage, tendon, and ligament). Ideally, a bioadhesive must be designed to match the material properties of the target tissue (e.g., elastic modulus of 10^2 - 10^4 Pa for soft connective tissue and $>10^4$ Pa for muscle, cartilage, and bone)²⁹. A tightly secured tissue repair will allow patients to undergo early, aggressive rehabilitation programs to promote regeneration of functional tissue, which is especially critical for relatively avascular tissues that are known to heal poorly otherwise. Bioadhesives with enhanced bulk mechanical properties also exhibit better adhesion performance³⁰. Additionally, it is important to control the swelling behavior of the cured

adhesive. Hydrophilic bioadhesives can potentially swell excessively, which is undesirable as it can lead to severe medical complications such as local nerve compression ^{31, 32}. Increased water content can also compromise the mechanical properties of the adhesive due to decreased crosslinking density ³³.

1.2.3 Biocompatibility and biodegradability of biomedical adhesives

One of the key criteria for a bioadhesive is that it needs to remain biocompatible during deployment as well as during its service life ³⁴. The typical curing process of an adhesive involves chemical crosslinking, and it is important that the release of reactive chemicals into the surrounding tissue is minimized. The introduction of the bioadhesive into the body is expected to generate an acute inflammatory response. However, it is not desirable for the bioadhesive to illicit sustained chronic responses ³⁵. In addition, unlike sutures or staples, adhesives can act as a barrier to the union of tissue surfaces. The ability of the bioadhesive to degrade with time as the tissue heals is important. However, the adhesive needs to maintain its mechanical integrity for a period of time immediately after surgery to prevent premature rupture of the repaired tissue. Degradation byproducts must be non-cytotoxic and, ideally, an adhesive should support rapid native cellular ingrowth and gene expression to regenerate functional tissue.

1.3 ROS production from Catechol adhesive

Catechol (1,2-dihydroxy benzene) is an organic compound that is commonly found in the DOPA molecule, which is secreted by mussel along with mussel adhesive proteins. In a

synthesis polymer preparation, dopamine moiety is commonly used to impart the catechol functionality onto various polymer backbones (i.e., polyacrylamide, PEG), resulting in the synthetic adhesive, which can perform under wet condition. However, the process gelation and crosslinking of such adhesive required periodate chemistry or other types of oxidants to facilitate the oxidation process of the catechol moiety (**Figure 3**). Catechol oxidized into quinone in the presence of O_2 resulted in superoxide formation leading to more oxidization of catechol through superoxide mediated oxidation. The superoxide mediated oxidation then oxidizes catechol to quinone but no longer generate superoxide but hydrogen peroxide instead. Since superoxide can also interact directly with protons,

Figure 3. $NaIO_4$ -mediated catechol oxidation to quinone and crosslinking. ROS, such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), are generated during the process. R = PEG polymer

more H_2O_2 and O_2 can be generated as a result. This chemical reaction loop will continue until no catechol is left to be oxidized. Since H_2O_2 half-life is significantly longer (hours vs. seconds) than those of superoxide, H_2O_2 is the main species of interest to control in order to tailor the biological response from catechol based adhesive³⁶.

1.4 H_2O_2 and its biological functions

H_2O_2 is a chemical substance that promptly used within living cells. It is known to govern many critical biological functions through the participation in oxidative biosynthesis functions in cells. Additionally, there is evidence pointing that cells exhibit a concentration-dependent response to H_2O_2 (**Table 1.**). At low concentration, it plays a crucial role as a signaling agent between cells. At an elevated concentration, it was observed to be serving as a defense mechanism for foreign invaders or kick start the inflammatory response. At a high concentration, H_2O_2 can break down biological tissues and denature proteins. H_2O_2 was utilized in biomedicine since the 1800s as biocidal agent³⁷. Recently, H_2O_2 (100 μM) was utilized not for their biocidal purposes but for wound lysate, induced angiogenesis, and allows new tissue growth³⁸. However, actively remove H_2O_2 by catalase enzyme delayed wound healing as eschar formation is disrupted. On the other hand, a higher dose of H_2O_2 ($\geq 105 \mu\text{M}$) retarded the wound healing and form a chronic wound as oxidative damage was done. However, at this concentration, H_2O_2 can achieve bacteriostatic³⁹. H_2O_2 concentration should be at least 106 μM in order to function as a bactericide agent⁴⁰. Sustaining release of H_2O_2 (10-10³ μM) from ascorbate acid selectively kill cancer cells but leave normal cells live⁴¹. More recently, a large dose

of 1 M was shown to completely kill both gram-positive and gram-negative bacterial as well as to deactivate both enveloped and non-enveloped virus⁴². Thus, the H₂O₂ concentration can be modulated to show different functions matched different applications and desired biological responses.

Table 1. H₂O₂ concentration and their correlated biological effect

[H₂O₂] (μM)	Biological Effect
Actively remove	Delayed dermal wound healing
10 ²	Delayed tenocyte proliferation
10 ² -10 ³	Accelerate dermal wound healing
10 ³	Bacteriostatic (E. coli)
10 ⁵	Delayed dermal wound healing
10 ⁶	Biocide and virucide
Sustain release of 10 ¹ -10 ³	Kill cancer cells but safe for healthy cells

1.5 H₂O₂ degradation and control method.

H₂O₂ degradation was done naturally within a living organism through the use of catalase enzyme. The enzyme is highly efficient and can break down H₂O₂ into H₂O and O₂⁴³. Catalytic degradation of H₂O₂ can also be achieved with a metal catalyst. Platinum, palladium, and iron are among the examples of metal groups that can catalytically breakdown H₂O₂^{44, 45}. However, metalloid oxides, SiO₂, was also found to be able to break down H₂O₂ in a similar manner to those of the metal oxides⁴⁴. The degradation rate of H₂O₂ with SiO₂ was reportedly slower by at least an order of magnitude (when compare to AlO₃) to up to 3 order of magnitude (when compare to ZrO₂) when compared to other conventional metal and ceramics⁴⁴. Decomposition is not the only mean of control for silica over H₂O₂, however. Multimember complexation between H₂O₂, SiOH, and H₂O was reported as a stable complexation which allows for a reduction in measurable H₂O₂^{46, 47}. The absorption was reported as reversible, which would allow the adsorbed H₂O₂ to participate in catalytic decomposition as the molecule of H₂O₂ are in close proximity of the silica-based material surface.

1.6 Silica particle as reinforcement and bioactive additives

The incorporation of inorganic nanoparticles (NP) into polymeric matrices provides a simple approach to create nanocomposites with improved mechanical properties and tailored biological responses^{48, 49}. This reinforcement strategy of using NP to form physical and chemical crosslinks within a polymer network has been rigorously studied in

the past decades. Specifically, nanoparticles used in biomedical applications include silica (Si) particle, bioactive glass, and hydroxyapatite ^{48, 50-52}. Among these particles, Si particles are one of the most commonly used, with key examples such as magnetoluminescence composites ⁵³, drug-releasing composite ⁵⁴, and composites with elevated mechanical property ^{49, 55}. Additionally, Si NP degrades into silicic acid, which has been found to be beneficial for bone and connective tissue formation and remodeling ^{56, 57}.

Despite the advantages associated with Si NP, some reported the toxicity of these particles due to its reduced size ^{56, 58, 59}. The cytotoxicity of NP is associated with the direct physical interaction between the particle and cell membrane, resulting in the cellular internalization of NP ⁶⁰. Particles with a larger size exhibited reduced cytotoxicity, as the internalization process is hindered when the particle size approaches micron scale ^{56, 58, 59, 61}. However, the performance of the micron-sized particle is inferior to that of NP in improving the mechanical properties of polymer matrices, due to a significant reduction in the surface area as well as the limitation in particle dispersion ^{62, 63}. To overcome the cytotoxicity associated with NP and the reduced performance of micron-sized particles as a filler, we prepared clustered microparticles (MP) that were formed through the clustering of NP. We hypothesized that the clustered MP would have reduced cytotoxicity due to its increased size while retaining nano-scaled surface features with an enhanced surface area for interacting with the polymer matrix (**Scheme 1**).

Scheme 1. Schematic representation of the chemical interactions involved in the curing and adhesion processes of the composite adhesive. PEG-DA contains catechol end-groups that can interact with silica nano- and microparticles (NP and MP, respectively) through strong reversible interactions (A). Oxidation of catechol in the presence of NaIO_4 forms highly reactive quinone, which results in catechol polymerization and curing of the adhesive (B), as well as the formation of an interfacial covalent bond with nucleophilic function groups (i.e., $-\text{NH}_2$ of lysine) and adhesion to the surface of soft tissue (C). Reproduced from reference 64 with permission from IOP Publishing copyright 2020.

1.7 Statement of purpose and experiment set-up

Mussel inspired catechol-based adhesive has gained popularity in research in recent years. However, only recently that our group has identified the byproduct to be singlet oxygen, superoxide, and H_2O_2 . It is crucial to understand their biological responses and able to has reasonable control over such a byproduct. H_2O_2 are the most stable reactive

oxygen species among all aforementioned all byproduct, and its biological effect is well understood. The concentration-dependent biological response of the H_2O_2 allows the unique approach in material design to take shape. Depending on the concentration and the exposure time, H_2O_2 can cause a variety of cellular and tissue responses ranging from accelerated wound healing (tissue-specific, ranging from 10^{-10} - $10^{-3}\mu\text{M}$) to tissue denaturation ($>10^4\mu\text{M}$). Therefore, H_2O_2 concentration can be modulated to fit different types of tissues and applications. One way of controlling hydrogen peroxide is through the use of silica particles. We first investigate and master our capability in controlling the particle synthesis before chemically altering the surface chemistry of the particle. Then the particle was subjected to complexation tests with H_2O_2 to determine their efficiency and affinity toward H_2O_2 control. We then study the use of silica particles as a modulator for H_2O_2 byproduct in the form of composite additives. The composite then subject to physical (i.e., gelation, rheometry, lap shear), chemical (i.e., Fourier Transform Infrared Spectroscopy, stability test), and biological (i.e., in vitro cell response) tests to determine and confirm the expected changes with the focus on the biological response. H_2O_2 -Silica particle complexation was found to be a viable option not only for the reduction of H_2O_2 but also for the unique property of highly hydrophilic silica particles, which allows the release of soluble silica out of the composite adhesive. The first approach for silica nanoparticle incorporated catechol-based bioadhesive was studied as a feasibility material model. The composite adhesive was utilized to evaluate the extent of control over H_2O_2 concentration and cellular responses. The study is also used to confirm that the addition of silica particles will not hinder the main function of the adhesive, which are remaining a

strong adhesive. Polyethylene glycol is selected as the backbone material because it has been approved by the FDA and was used in many applications, including cell encapsulation⁶⁵, scaffolding⁶⁶, and drug delivery⁶⁷. Silica was thought to be bioinert or cytotoxic. However, recent understanding shines a new light on the material. Crystallinity⁵⁹, size⁵⁸, and surface charge⁶⁸ of the particle govern their reactivity with living cells. Combining with PEG hydrogel, they are ideal biocompatible composite systems that the biological responses to the hydrogel can be fully contributed to the H₂O₂ released during catechol autoxidation and the resulting modulation of H₂O₂ from the particle. It is clear from the previous study that adding particle alone has limited use when specific applications required a higher concentration of H₂O₂ (i.e., antimicrobial, dermal wound healing). As a result, the continuation study was utilizing the robust design of experiment approach to predict and narrow down the formulation to be tested for, specifically, dermal wound healing application. Full thickness wound model in mice was selected as the model to study the effect of adhesive architecture, H₂O₂ concentration, and particle incorporation on the wound healing process. With more flexible formulation selection, silica particle role was changed from scavenger in the previous study into a complete modulator allowing for the fine tuning of H₂O₂ to be achieved. Finally, the wound healing response to the treatment of the adhesive and their composites were determined.

2 Synthesis, Modification, and Characterization of Silica Particle and Catechol-Based Polyethylene Glycol Adhesive²

2.1 Synthesis, Modification, and Characterization of Silica Particle

2.1.1 Understanding Silica Particle and Their Biological Effect

Two of the most commonly used silica precursors in both industry and research include tetraethyl orthosilicate (TEOS), tetramethyl orthosilicate (TMOS), and sodium silicate (Na_2SiO_3) or water glass⁶⁹⁻⁷¹. All silica precursors produce the final product through the process of hydrolysis and condensation of silicic acid precursors under catalysts resulting in predominantly silicon dioxide product⁷². Both acidic and basic conditions can be used to catalyze the reaction as protonation of alkoxide can occur faster with the extra protons from the acid species during the hydrolysis process (**Figure 4**)^{70, 72}. However, the extra protons in from the acid species also allow the interaction between silanol groups and ethoxy groups to occur, slowing down the condensation process. Ultimately, the condensation between the silanol groups or between silanol groups and ethoxy groups creates siloxane bridges (Si-O-Si) that form the entire silica structure⁷². The inverse relationship is true for the basic catalytic reaction. Hence, the acid catalyst will result in

² Parts of the content in this chapter was published in *Biomedical Materials*, 13(2), p.025003. Copyright 2020 American Chemical Society. <https://iopscience.iop.org/article/10.1088/1748-605X/aa985d/meta>

fast hydrolysis and slow condensation. Inversely, the basic catalyst will result in slow hydrolysis and fast condensation.

The main difference between all silica precursors is the rate of chemical conversion, which results in differences in the rate of silica product formation through hydrolysis and other preparation methods (i.e., pyrolysis, acid precipitation, etc.) (**Figure 4**)^{72, 73}. For instance, TMOS demonstrated to be around four times faster hydrolysis rate when compare to TEOS is an alkoxy preparation method^{72, 73}.

TEOS and TMOS are effective silica precursors with generally higher than 90% recovery rate of silica product by weight when preparing through hydrolysis and more than 70% recover rate by weight when pyrolytically preparing through flame spray pyrolysis process (FSP)^{72, 74}. FSP is the most commonly utilized method to produce silica on an industrial scale as there are no solvent and separation required to retrieve the final product⁷³. However, Na_2SiO_3 is more commonly used, and the resulting silica particle prepared from the FSP method has high crystallinity as high heat exposure is required for such synthesis method⁷⁵.

Crystallinity is found to be one of the deciding factors in utilizing silicon-based particle as crystalline particle both synthetic (silica nanocrystals) and natural (i.e., mica, asbestos, and silicon fume) are all found to cause cell apoptosis and can leads to tissue degeneration especially in epithelium cells and respiratory tracts^{58, 59}. Additionally, crystallinity is also directly governing the mode of which the particle causes a biological response from highly toxic to cells to nearly biocompatible with higher crystallinity

resulting in higher toxicity^{58, 64}. It is also a deciding factor whether the particle can be biologically degraded through hydrolysis as the lower

Figure 4. Basic-catalyzed silica hydrolysis and condensation (A) and acid-catalyzed silica hydrolysis and condensation. Reprint from ref. 71 and 69, with permission from Elsevier copyright 2020 and The Royal Society of Chemistry, respectively

crystalline particle can partially degrade through hydrolysis before being ingested by the cells ^{54, 56, 59, 76}. There are studies suggested that the degraded product of the amorphous silica particle is silicic acid, which can contribute to increased cell proliferation and cell migration ^{56, 76}. As a result, most research in biomedicine, when utilized silica-based materials, are willingly lowering crystallinity with a compromise on the use of solvent and separation technique to ensure an improved biological response.

2.1.2 Stöber Synthesis Method

In 1968, Werner Stöber and his research team had reported a new approach in synthesizing particulate in an aqueous reactor with ammonia as a catalyst to control the hydrolysis and condensation kinetics of ethoxy silane in alcohol ^{70, 77}. The resulting product is a fine powder of uniform silica particles (**Scheme 2**). For Stöber synthesis, morphological and size distribution controlled are the main benefit of getting from the synthesis method. The key synthesis methods derived from Stöber finding may include Bogush and Zukoski ⁷⁴. The investigation of factors that affect the particle formation in this synthesis method may include a selection of silica precursors, the use of surfactant, application of shear force, pH, and temperature of the reactor solution^{70, 72, 78, 79}. These factors are not only affecting the final product, the particle but also affect the reaction speed, separation method, purification method, etc. The modified Stöber method utilized the understanding of these factors and modified the synthesis setup, so the researcher has more control than the initial concept proposed by Stöber and his colleagues.

Scheme 2. representation of simplified Stöber synthesis. Reproduce from Ref. ⁷⁰ with permission from Elsevier copyright 2020

2.1.3 Exploratory Experiment on Modified Stöber Synthesis Method

2.1.3.1 Material

Tetraethyl orthosilicate (TEOS, 99.8%), methanol, ethanol (200 proof), potassium hydroxide (pallet, 85%), sodium hydroxide (NaOH, 50% w/w), cetrimonium bromide (CTAB, 99.9%), acetic acid (CH₃COOH, glacial 98%), and hydrochloric acid (HCl, 37%) were purchased from Fisher Scientific (Pittsburgh, PA).

2.1.3.1 Method

The synthesis setup consisted of a hot plate stirrer with digital temperature and speed indicator (Fisher Scientific), Erlenmeyer flask with appropriate volume (100-500 ml), a glass flask adapter (24/40), and rubber caps. A glass reflux column setup was utilized during the purification and surface modification to contain escaping acidic fume (**Figure5**). Aluminum foil was used to create a localized heat radiator to improve temperature management. For the basic catalyst experiment, 12.5 ml of NaOH was added to 250 ml of ethanol/deionized water mixed solution (1:1 v/v) under vigorous stir to create a basic reactor solution. For the acid catalyst experiment, 20 ml of HCl was added to 250 ml of ethanol/deionized water mixed solution (1:1 v/v) under vigorous stir to create an acid reactor solution. 9.7 ml of TMOS and 10.6 ml of TEOS (1g) was added to the reactor solutions dropwise. The mixed solution was allowed to react for 1 or 12 hours before the solution was collected and filtered through a paper filter with a pore size of 40 μm to remove large contaminants and aggregates. The filtered solution was then transferred to a centrifuge tube. Ultracentrifugation (10000 Gs) was utilized to collect the particle. The filtered solution was centrifuged for 15 minutes; then the supernatant was discarded. The particle was then resuspended in 40 ml deionized water to remove residual acid or base. The purification was repeated three times. The resulting particle was then dried under vacuum for 48 hours before weighting to find the recovery rate. The recovery rate was calculated via the following equation:

$$\%R = 100 \times W_1 \div 0.461 \quad (1)$$

Where %R is the recovery rate of the particle, and W_1 is the weight of the dried particle. 0.461 was derived from the precursor silica conversion as 1 g of silica precursor would result in 0.461 g of SiO_2 with 100% conversion efficiency

Figure 5. Equipment setup for silica particle surface modification. Yellow box indicates the flask convertor and reflux column connection

Table 2. Exploratory silica particle synthesis condition

Silica Precursor	Condition	Time (hours)	Approx. Recovery (%)
TMOS	pH 1 25°C 400 rpm	1	30

	pH 11 25°C 400 rpm	12	66
		1	75
		12	84
TEOS	pH 1 25°C 400 rpm	1	12
		12	65
	pH 11 25°C 400 rpm	1	64
		12	77

The results indicated that TMOS is a more efficient silica precursor as it can form silica particles more rapidly when compared to TEOS (**Table 2**). However, TMOS produced a higher amount of aggregates as well as was found to generally be harder to control morphology and size^{80, 81}. As a result, TEOS was selected to be the main silica precursor in this study.

As a continuation in particle synthesis, nonporous silica nanoparticle (NSi) was prepared using a modified Stöber method without surfactant⁶⁴. The silica precursor solution was prepared by mixing 4 g of TOES with 50 mL of ethanol and sonicated for 10 minutes. The reactor solution was prepared with 25 mL deionized water mixed with 30 mL ethanol and vigorously stirred (15 minutes at 400rpm) using a hot plate stirrer at 75°C. The pH of the reactor solution was maintained at pH 11 by adding 0.1 M NaOH. The silica

precursor solution was added dropwise into the reactor solution at a rate of approximately 300 $\mu\text{l}/\text{second}$ while stirring at 400 rpm. The solution turned milky white immediately after the precursor have been added to the reactor solution. After stirring for 1 hour at 75 $^{\circ}\text{C}$, the pH of the reaction mixture was neutralized by adding 0.1 M acetic acid. The reaction mixture was further stirred for an additional 12 hr at room temperature. The reaction mixture was filtered using a filter paper with a mesh size of 20 μm to remove large contaminants. Ultra-highspeed centrifugation (10000 Gs) was used to separate the particles from the solution. The particles were washed vigorously three times with ethanol to ensure complete dehydration and removal of sodium acetate. After drying under a vacuum of 24 hrs, the yield of NP was 1.5 g. Mesoporous silica particles with low (LSi) and high (HSi) porosity were prepared using the same Stöber method with CTAB as a surfactant. The silica precursor solution was prepared by mixing 0, 9.4, or 18.8 g of CTAB (for NSi, LSi, and HSi, respectively) into 500 ml of ethanol and water mixed solution (2:1 v/v ratio) and vigorously stirred (15 min at 400 rpm) at 55 $^{\circ}\text{C}$ via a heating manifold and pH 11 by adding 0.1M NaOH dropwise. Then, 10 ml of TEOS solution was added dropwise into the reaction solution at a rate of approximately 300 $\mu\text{l s}^{-1}$ while stirring at 1000 rpm. The mixed solution was left stirring for 1 hour at 55 $^{\circ}\text{C}$ and pH 11, then the heating manifold was removed, and the pH of the reaction mixture was neutralized by adding 0.1M HCl dropwise. The mixture was further stirred for an additional 15 minutes at room temperature. The reaction mixture was centrifuged at 10,000 kGs then washed three times with methanol, ethanol, and deionized (DI) water. The particles were dried under vacuum for 24 hours and sifted using American Society

for Testing and Materials (ASTM) 450 (32 μm) sifters to remove any large contaminants and aggregates. The yield of the NSi, LSi, and HSi particles was 75%, 68%, and 66%, respectively.

2.1.4 Surface modification

Silica and silicate surface modification can easily be done with various chemicals (i.e., acid and alkaline treatment), mechanical (mechanical abrasion), and physical treatments (i.e., increase temperature). Here we will specifically discuss chemical treatment of the silica surface to produce hydroxide sites on the silica surface

The chemical treatment on silica surface are widely used in electronics and industry during cleaning and activating the surface for further modification with silane coupling chemistry. However, most of the cleaning and surface treatment techniques require the use of strong alkaline treatment (i.e., potassium hydroxide (KOH), sodium hydroxide (NaOH)), strong acid treatment (i.e., hydrogen fluoride (HF)). These are highly hazardous materials which require special handling and disposal as they are highly corrosive and reactive with water. Exploratory experiments were done to functionalized the surface of silica particle synthesized from the previous section with strong alkaline. However, the total loss of material was observed (**Table 3**). The treatment was done with 100 mg of HSi particle in 5 ml of KOH, NaOH, and HCl solution under a vigorous stir. A borosilicate glass reflux column was used with the experiment with a heated solvent to prevent alkaline fume escapes. The solution was left stirring at 400 rpm at room temperature or at 95 °C. After 0.5 or 6 hours, the solution was then allowed to cool down

to room temperature then be transferred to a centrifuge tube. Ultracentrifugation (10000 Gs) was used to collect the particle. The centrifugation was done for 15 minutes, then the supernatant was discarded, and the particle was resuspended in 40 ml deionized water. The resuspended solution was then centrifuged to remove residual acid or base from the particle, repeating three times. The purified particle then dried under vacuum for 48 hours before being weight again to determine the recovery rate. The following equation was used to calculate the recovery rate in percent:

$$\%R = 100 \times W_1 \div W_0 \quad (2)$$

Where %R is recovery rate in percent, W_1 is the weight of the particle after treatment, and W_0 is the initial weight of the particle

Table 3. Exploratory surface modification condition

Reactor	Concentration	Time (hours)	Temperature	Approx. Recovery
KOH	0.1 M (pH 13)	0.5	25	<40%wt
		6	95	Loss
	1 M (pH 14)	0.5	25	Loss
		6	95	Loss
NaOH	1 M (pH 13)	0.5	25	<20%wt

	10 M (pH 14)	6	95	Loss
		0.5	25	Loss
		6	95	Loss
HCl	0.1 M (pH 1)	0.5	25	94%
		6	95	87%
	1 M (pH 0)	0.5	25	95%
		6	95	85%

The recovery rate was found to be low, and the separation was found to be poor as the resulting particle are presumably lost a large amount of mass and integrity with the strong alkaline treatment. As an alternative, weaker acid has been utilized in place of a strong acid with elevated temperature. HCl treatment was found to mildly affect the particle recovery and deemed suitable for surface modification of the nanoparticle. This is further supported by the fact that silica material has an isoelectric point at around pH 2, as shown in **Figure 6**. Lower and higher pH value allows the dissolution of silica to occur at various speed. The dissolution kinetics of silica was found to be the highest in the alkaline range (pH>10) and, though significantly lower, highly acidic condition for the acidic range (pH<3). The HF treatment was never attempted as it is generally known that

HF reacts more violently to glass than KOH as well as the special hood is required to safely handle HF.

Figure 6. Effects of pH value on the silica condensation rate, charge properties, and charge density on the surface of the silica species. Reproduced from Ref. 72 with permission from The Royal Society of Chemistry.

2.1.5 Characterization methods

The most important aspect of utilizing Stöber method is to ensure minimal crystallinity and the ability to effectively control the size and morphology of the resulting particle. The particle characterization method is divided into two approaches, which generally include physical and chemical analysis. The particle chemical properties are usually determined with simple FTIR analysis to ensure the presence of Si-O-Si, Si=O, and Si-

OH. The FTIR is also utilized to ensure the efficacy of the purification method as well. The physical properties of silica particles are generally involved in morphological analysis and size distribution analysis. This is to ensure that the particle size and morphology are consistent among the sample group. X-ray diffraction was used to ensure the amorphous nature of the particles (**Figure 7**). There were no definitive peaks that are correlated with the crystalline SiO_2 standard. The results indicated that all particles prepared with the modified Stöber method are amorphous regardless of the use of surfactant and surface modification.

Figure 7. XRD spectra of HSi, AHSi, LSi, and NSi. Pink markings indicate correlated crystalline SiO_2 crystal spectra, cristobalite

3 Controlling the Release of Hydrogen Peroxide from Catechol-Based Adhesive Using Silica Nanoparticle³

3.1 Abstract

Catechol-based bioadhesives generate hydrogen peroxide (H_2O_2) as a byproduct during the curing process. H_2O_2 can have both beneficial and deleterious effects on biological systems, depending on its concentration. To control the amount of H_2O_2 released from catechol-containing polyethylene glycol-based adhesive (PEG-DA), the adhesive was formulated with silica nanoparticles (SiNP) prepared with increased porosity and acid treatment to increase Si-OH surface content. These SiNP demonstrated increased surface area, which promoted interaction with catechol and resulted in increased cure rate, bulk mechanical properties and adhesive properties of PEG-DA. Most importantly, SiNP demonstrated a 50% reduction in the released H_2O_2 while improving the cell viability and proliferation of three primary cell types, including rat dermal fibroblasts, human epidermal keratinocytes, and human tenocytes. Additionally, SiNP degraded into soluble Si, which also contributed to increased cell proliferation. Incorporation of porous and acid-treated SiNP can be a useful approach to simultaneously modulate the concentration of H_2O_2 while increasing the adhesive performance of catechol-based adhesives.

³ This article was *just accepted* in *ACS Biomaterials Science & Engineering*. Copyright 2020 American Chemical Society. <https://pubs.acs.org/doi/abs/10.1021/acs.biomac.7b01311>

3.2 Introduction

Mussels adhere strongly to various surfaces under wet conditions through the secretion of adhesive proteins that solidify to form adhesive plaques¹⁶. Catechol moiety found in mussels adhesive proteins (MAPs) is one of the main contributors to the interfacial binding and crosslinking capabilities of these proteins⁸². Catechol-modification of natural and synthetic polymers is widely used to create in situ curable bioadhesives with strong, wet adhesive properties^{83, 84}. To activate catechol for crosslinking, catechol needs to be oxidized into its reactive quinone form by chemical-mediated (i.e., periodate) or enzymatic (i.e., peroxidase) oxidation^{85, 86}. This is a critical step to initiate cohesive and interfacial crosslinking of catechol-containing adhesive. Specifically, periodate is one of the most commonly utilized oxidants for initiating curing of injectable biomaterials⁸⁷⁻⁸⁹. Recently, our lab reported that hydrogen peroxide (H_2O_2) is generated as a byproduct during the oxidative crosslinking and curing of catechol-containing adhesive⁴⁰. The generated reactive oxygen species (ROS) contributed to localized in vitro cytotoxicity, promoted antioxidant enzyme expression in primary dermal and tendon fibroblasts, and promoted M2 macrophage polarization when the adhesive was implanted subcutaneously.

Living organisms naturally generate H_2O_2 as a part of normal wound healing and inflammatory response⁹⁰⁻⁹². The effect of H_2O_2 on biological systems is also highly concentration-dependent. Application of H_2O_2 at micromolar ranges have been shown to accelerate dermal wound healing^{93, 94}. Additionally, H_2O_2 is a natural disinfectant³⁹. However, high levels of H_2O_2 can destroy healthy tissues, lead to chronic wounds, retard

natural healing cascade, and induce tumor formation ^{38, 95}. Given the varying H₂O₂-associated biological responses, there is a need to tune the amount of H₂O₂ released from catechol-containing biomaterials according to the application-specific material design.

H₂O₂ concentration can be controlled through the use of catalytic metal oxides (Al₂O₃, TiO₂), metal ions (Fe³⁺, Mn²⁺, and Cu²⁺) and enzymes such as catalase ^{40, 45, 96}. However, most catalytic metal oxides and ions are not suitable for biological applications due to their cytotoxicity, and toxic metal accumulation can occur with prolonged exposure ^{68, 97}. Although catalase readily decompose H₂O₂ and are suitable for biomedical applications, they are limited by elevated costs and issues with stability ^{98, 99}. As an alternative, silica (Si) particles and silicate material are economically viable and can degrade H₂O₂ ⁴⁴. Si materials are more biocompatible when compared to other metal oxides ⁹⁷. Additionally, H₂O₂ forms a multimember ring complex with Si-OH groups on the surface of Si-based material in the presence of water, which helps to further stabilize the complex ^{100, 101}. However, Si material decomposes H₂O₂ at a significantly slower rate when compared to other metal oxides ⁴⁴. Since both degradation and complexation of H₂O₂ on Si material are surface dependent phenomena, a combination of increased surface area and surface modification techniques can potentially improve the efficiency of silica particles for H₂O₂ degradation.

In this work, we prepared highly porous Si particles and further treated these particles with strong acid to create a Si-OH-rich surface. We hypothesize that the combination of increased surface area and surface chemistry modification can be utilized to modulate the

3.3 Materials and methods

3.3.1 Materials

Phosphate buffer saline (PBS), tetraethyl orthosilicate (TEOS, 99.8%), methanol, ethanol (200 proof), sodium hydroxide, cetrimonium bromide (CTAB, 99.9%), hydrogen peroxide solution (H_2O_2 , 30%), dimethyl sulfoxide (DMSO), and hydrochloric acid (HCl, 37%) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium periodate (NaIO_4 , >99.8%) was purchased from Acros Organics (Fair Lawn, NJ). Pierce Quantitative Peroxide Assay Kit with sorbitol (ferrous oxidation-xylenol orange (FOX) assay), fetal bovine serum (FBS), and Penicillin–Streptomycin (10 U/ml) were purchased from Thermo Scientific (Rockford, IL). Rat primary dermal fibroblasts (RDF) were purchased from Cells Biologics (Chicago, IL). Dermal Cell Basal Medium, keratinocyte growth kit, primary human tenocytes (HT), and primary human epidermal keratinocytes (HEK) were purchased from ATCC (Manassas, VA). TransWell® Permeable Supports (12 mm insert, 12 well plate, 3.0 μ , polycarbonate membrane) were purchased from Corning Costar (Pittston, PA). Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/L glucose and glutamine, without sodium pyruvate), and trypsin–EDTA (0.05% Trypsin/0.53 mM EDTA in Hank's balanced salt solution) were obtained from Corning Cellgro (Manassas, VA). Tenocyte Growth Medium was purchased from Zenbio (Research Triangle Park, NC). Polyfreeze®, phosphate-buffered saline (PBS, BioPerformance certified, pH 7.4), bovine liver catalase, Trichrome Stain (Masson's) Kit, Bouin's solution, and Weigert's iron hematoxylin solution were purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 98% (MTT) was purchased from Alfa Aesar (Ward Hill, MA). Anti-Ki-67 antibody (ab15580), goat anti-rabbit IgG H&L (Alexa Fluor 488) (ab150077), and goat anti-rabbit IgG H&L (Alexa Fluor 647) (ab150079) were purchased from Abcam (Cambridge, MA). 4,6-Diamidino-2-phenylindole (DAPI) was obtained from Invitrogen (Grand Island, NY). PEG-DA was prepared following previously published protocols using an 8-armed PEG (MW=20 kDa, JenKem USA, TX) ⁶⁴

3.3.2 Synthesis of Silica Particles

Nonporous silica nanoparticle (NSi) was prepared using a modified Stöber method without surfactant ⁶⁴. Mesoporous silica particles with low (LSi) and high (HSi) porosity were prepared using the same Stöber method with CTAB as a surfactant. The silica precursor solution was prepared by mixing 0, 9.4, or 18.8 g of CTAB (for NSi, LSi, and HSi, respectively) into 500 ml of ethanol and water mixed solution (2:1 v/v ratio) and vigorously stirred (15 min at 400 rpm) at 55 °C using a heating manifold and maintained at pH 11 with dropwise addition of 0.1M NaOH. Then, 10 ml of TEOS solution was added dropwise into the reaction mixture at a rate of roughly 300 $\mu\text{l s}^{-1}$ while stirring at 1000 rpm. The mixture was left stirring for another hour at 55 °C. 0.1 M HCl was added dropwise to the reaction mixture to neutralize its pH, which was further stirred for another 15 minutes at room temperature. The reaction mixture was centrifuged at 10,000 kGs then washed 3 times with methanol, ethanol, and deionized (DI) water. The particles were dried under vacuum for 24 hours and sifted using American Society for Testing and

Materials (ASTM) 450 (32 μm) sifters to remove any large contaminants and aggregates. The yield of the NSi, LSi, and HSi particles was 75%, 68%, and 66%, respectively.

3.3.3 Silica Particle Modification

Acid-treated HSi (AHSi) particles were prepared using a modified version of a previously developed acid activation technique ⁷⁹. HSi (1 g) was resuspended in 200 ml of ethanol, then 2 ml of 12 M HCl was added into the mixture. The mixture was heated at 90 °C with continued stirring under a N₂ environment with a reflux column for 12 hours. NaOH (17.6M) was added dropwise to neutralize the solution pH to 6.5 and then was further stirred for 30 minutes at room temperature. The mixture was centrifuged at 10,000 kGs at 40C, and the particles were washed 3 times with methanol, ethanol, and then DI water. The particles were dried under vacuum for 24 hours and sifted through ASTM 450 (32 μm) sifters. The yield of these particles was approximately 33%. The particles were dehydrated in a 200°C oven over a bed of potassium carbonate desiccant for 12 hours before backfilling with N₂ gas to remove all moisture.

3.3.4 Silica Particle Surface and Composition Characterization

The total surface area of the particle was calculated using the Brunauer-Emmett-Teller (BET) model. A BET surface area Micromeritics ASAP 2000 sorptometer (Micromeritics) was utilized to measure the surface areas of silica particles by nitrogen adsorption at liquid-nitrogen temperature (-196.15°C). The samples were degassed at 120 °C for 12 hours before the surface measurement. The total surface area was determined

by the physical absorption of N₂ gas on to the particle surface ¹⁰². A FEI 200kV Titan Themis. Scanning Transmission Electron Microscope (S-TEM) was used to visualize the morphology of the particle, and Energy-dispersive X-ray spectroscopy (EDS) was used to measure the elemental (Si and O) content of the particle. ImageJ program (NIH) was used to measure the particle size from TEM images.

3.3.5 Adhesive Formation

The PEG-DA adhesive hydrogels were prepared following a previously published protocol ⁶⁴. The adhesive was prepared by mixing equal volume of the adhesive precursor solution (300 mg ml⁻¹ of PEG-DA in 2xPBS at pH 7.4) and NaIO₄ solution (54.5 mM in DI water). The final concentration of PEG-DA was 150 mg ml⁻¹. The Si particle incorporated composite adhesives were formed in a similar manner with the addition of 20 wt% of the corresponding Si particles into the PEG-DA precursor solution. The final concentration of the particles in the composite adhesive was 10 wt%. The final NaIO₄ to dopamine molar ratio was 0.5 for all adhesive formulations. To determine the gelation time of the hydrogels, 200 µl of the mixed solution was introduced into a 1.5 ml scintillated vial. The time it took for the mixture to cease flowing in a tilted vial was determined as the cure time ²⁷. Unless specified otherwise, the adhesive was cured in between two borosilicate glass sheets with a silicon rubber mold (width = 4 cm, height = 4 cm, thickness = 1.5 mm) for 24 hours. The cured adhesive sheet was cut into disk shapes using a tissue punch with a diameter of 10 mm to create hydrogel disks with a diameter of 10 mm and a thickness of 1.5 mm. The hydrogel disks were further

equilibrated in PBS (pH = 7.4) before additional characterization. Adhesive formulations containing NSi, HSi, and AHSi are denoted as PEG-NSi PEG-HSi and PEG-AHSi, respectively.

3.3.6 Physical and Chemical Characterization of the Adhesive

Samples were freeze-dried for at least 48 hours and characterized using Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer Spectrum One with DLATGS detector) with a single bounce attenuated total reflectance (ATR) unit (PIKE Technologies, diamond internal reflection element (IRE) GladiATR). The scan rate was set at 800 scans/minutes with a resolution of 1 cm⁻¹. The FTIR experiments were done at ambient conditions. In vitro degradation behavior of adhesive disks (n = 3) was determined by incubating the samples in 5 ml of PBS (pH = 7.4) at 37 °C and tracking their remaining mass (M_r) over 8 weeks. The hydrogel discs were equilibrated overnight before the degradation experiment started to ensure stable initial weight. The discs were then dried under vacuum for at least 48 hours. Dry weight was used to determine the degradation rate using the following equation:

$$M_r = \frac{(M_0 - M_t) \times 100}{M_0} \quad (3)$$

where M₀ and M_t are the initial mass of the adhesive and its mass after incubation for a given amount of time, respectively ⁸⁶. The PBS solution was replaced every 7 days.

To determine the concentration of soluble silica released from the adhesive, fully cured samples ($n = 3$ for each group) were incubated in 1 mL of 1xPBS (pH 7.4) in a polystyrene container for 2 weeks. The PBS extracts were diluted by adding deionized water to 10 mL in a polystyrene centrifuge tube prior to testing. The diluted extract was then evaluated for silica content using inductively coupled plasma optical emission spectroscopy (ICP-OES; Perkin Elmer Optima 7000DV) utilizing a cross-flow nebulizer (plasma 15 mL min⁻¹, auxiliary 0.2 mL min⁻¹, nebulizer 0.80 mL min⁻¹, RF power 1,350 W, pump speed 1.5 mL min⁻¹) Instrument Calibration Standard 3 (Perkin Elmer) was used to create the calibration standards. Three samples per formulation were collected and each sample was analyzed three times.

An oscillatory rheometer (Discovery Hybrid Rheometer HR2; TA instruments, Newcastle DE) was used to determine the storage (G') and loss (G'') moduli of the cured adhesive samples. Strain sweep experiment was performed by subjecting disk-shaped samples ($n = 3$) to a strain of 0.01–100% at a frequency of 0.1 Hz using a parallel plate setup at ambient temperature. The gap distance was set at 87.5% of the thickness of each individual hydrogel, to ensure sufficient traction during the rheometry experiment. 1 mL of pH 7.4 PBS was introduced around the composite adhesive to prevent dehydration. Lap shear adhesion test was performed using bovine pericardium while following a previously published protocol according to ASTM standard F2255-05⁶⁴. Strips of pericardium tissues (2.5 cm x 2.5 cm) were kept moist in PBS. 100 μ L of PEG-DA precursor solution and NaIO₄ solution with and without Si particles were mixed in a

scintillated glass vial before being transferred on to the surface of the tissue. Another piece of pericardium tissue was applied over the adhesive to create an adhesive joint (overlapping area of 2.5 cm²), which was compressed with a 100-g weight for 15 min. The adhesive joint was submerged in PBS (pH = 7.4) at 37 °C for overnight prior to testing. A digital caliper was used to measure the dimensions of the overlapped area of each adhesive joint before testing. The fixation of the tissue pieces on to the test stage was done with serrated metal grips to ensure sufficient traction. An Electroforce® machine (Bose Electroforce Group, MN) was used to pull apart the adhesive joint to failure at a rate of 0.1 mm s⁻¹. The adhesive strength was determined by the maximum load divided by the initial overlapped area of the adhesive joint.

3.3.7 In vitro Cell Viability

The cell viability experiment was performed following a previously published protocol with modifications ⁶⁴. A quantitative MTT assay was used to determine the relative viability of cells exposed to the adhesives and their extracts, while following ISO 10993-5 guideline ¹⁰³. Microfiltration was utilized as a sterilization method. The PEG-DA precursor solution and NaIO₄ solution were filtered with a 0.2 µm polyethersulfone syringe filter (VWR International, USA). The particles were sterilized using ethylene oxide in a microcentrifuge tube. Adhesive extracts were prepared by forming hydrogel discs (diameter =10 mm and thickness = 1.5 mm, n=3) as described above, followed by incubating each disc in 1 ml of cell-specific culture medium for 24 hours at 37°C. RDF, HEK, and HT were suspended in their cell-specific culture medium. Cell suspensions

were then added to a 96-well microculture plate at a density of 1,000, 2,000, and 2,000 cells/well, respectively. Varying amount of culture medium was added to each well to maintain the culture medium volume of each well to 100 μ l. The cells were incubated in a humidified incubator (37 °C, 5% CO₂) for 24 hours to obtain a confluent monolayer of cells. A light microscope (Olympus, PA) was used to observe the monolayer to ensure the confluence level. Then, the medium was replaced with 100 μ l of the adhesive extract. After incubation for 24 hours, the medium was removed and replaced with 50 μ l of MTT solution (1 mg ml⁻¹ in pH 7.4 PBS) and further incubated for another 2 hours. After the 2-hour incubation, the MTT solution was replaced by 100 μ l of DMSO to dissolve formazan crystals. The absorbance of each well was measured at 570 nm (reference 650 nm) using a Synergy HT Multi-Mode Microplate Reader (BioTek, USA). The relative cell viability was calculated by dividing the average absorbance value of the sample from that of cells cultured in the culture medium after subtraction of the absorbance background of untreated culture medium ⁶.

3.3.8 In vitro Cell Proliferation

All experiments were performed with a Transwell set up to ensure the continuous exposure of the cells to the composite adhesive. Ki-67 and DAPI fluorescence staining were performed to analyze the proliferation of cells exposed to the adhesive placed in Transwell inserts. NaIO₄ (160 μ l) and sterile adhesive precursor solution (1:1 v/v) was introduced into a circular silicone mold with a dimension of 10 mm x 1 mm. The mixed solution was left to gel for 5 minutes before being transferred into Transwell inserts.

RDF, HEK, and HT were seeded into 12-well Transwell-compatible plates at a density of 5,000, 20,000, and 20,000 cells/well, respectively. The cells were incubated (37 °C, 5% CO₂) for 24 hours to obtain a 60-75% confluent monolayer of cells. A light microscope (Olympus, PA) was used to observe the monolayer to ensure the confluence level. The culture medium was removed before the Transwell inserts were introduced. Fresh medium (1ml) was added. Transwell inserts were removed after 24 hours of incubation along with the culture medium. Cells were fixed using a 1% paraformaldehyde solution for 15 minutes and permeabilized using a 1% Tween20 solution. The solution was removed, and the cells were washed with a treatment solution of 0.1% Triton X-100. After the treatment solution was removed, the primary Ki-67 antibody solution (1:1,000) was introduced and incubated at room temperature for 45 minutes before being washed 3 times by the washing medium for 5 minutes each. The washing medium was removed, then Alexa Fluor 488 green fluorescence stain was introduced for 45 minutes before being wash 3 times with the washing medium for 5 minutes each. The washing medium was removed, and the DAPI solution (1:1,000) was introduced for 5 minutes before being washed 3 times using the washing medium for 5 minutes each. The washing medium was removed, and the cells were analyzed via fluorescence microscopy using an automated microscope (EVOS, Fisher). Ki-67 positive cells and cell nuclei fluoresced green and blue, respectively. The total cell counts were averaged from all 27 images of each formulation.

3.3.9 Statistical analysis

Statistical analysis was performed using SigmaPlot 14 software. One-way analysis of variance (ANOVA) with Tukey's HSD analysis and Student's t-test were performed for comparing means of multiple groups and two groups, respectively, using a p-value of 0.05.

3.4 Results and discussion

3.4.1 Characterization of Si Particles

S-TEM was used to determine the particle size and morphology of each type of silica nano particle (SiNP) (**Figure 8**). NSi appeared dark with solid-like morphology. On the other hand, LSi, HSi, and AHSi were prepared with a quaternary ammonium surfactant, CTAB, and appeared lighter in color with porous structures. The particle size of NSi averaged around 660.6 ± 52.63 nm, which was larger when compared to the porous particles (528.08 ± 34.25 nm and 422.24 ± 23.24 nm for LSi and HSi, respectively) (**Figure 9**). This result suggests that the incorporation of CTAB reduced the final particle size by reducing the microbubble size in the reaction solution through surface energy reduction^{72, 78}. In the case of AHSi, acid treatment did not significantly affect its particle size when compared to HSi.

BET-N₂ sorption measurement demonstrated that the addition of CTAB during synthesis increases the total surface area of the particle (**Figure 10**). NSi exhibited the smallest

surface area ($13.72 \pm 0.26 \text{ m}^2/\text{g}$) among all the SiNP, while the porous SiNP exhibited an order magnitude higher surface area (256.58 ± 0.71 , 327.45 ± 5.51 , and $386.75 \pm 5.19 \text{ m}^2/\text{g}$ for LSi, HSi, and AHSi, respectively). CTAB and other surfactants introduce porosity into the silica particle, creating a mesoporous particle as a result ^{72, 78}. With an increasing amount of CTAB, the porosity increased as more porogen were introduced. Acid treatment after particle synthesis also significantly increased the pore volume in AHSi. The effect is directly linked to the removal of silicate groups from the particle surfaces as colloidal silicates during acid treatment ¹⁰⁴. Hence, the newly exposed surface formed a multitude of hydroxylated silica groups under acid treatment ^{104, 105}. S-TEM EDS analysis indicated that the composition of silica to oxygen ratio (S:O) decreased when HSi was treated with acid. The higher oxygen (O) content in AHSi indicated an increase in oxygen-silicon groups (i.e., Si=O, Si-OH, Si(OH)₂, and Si(OH)₃) on the surface of the particle.

Figure 8. STEM images of non-porous silica nanoparticle (NSi) (A), porous silica nanoparticle (LSi) (B), highly porous silica nanoparticle (HSi) (C), and acid-heat treated highly porous silica nanoparticle (AHSi) (D). Scale bars are 50 nm.

Figure 9. Particle size distribution of silica particles. * $p < 0.05$ when compared to each other.

Figure 10. Surface area of Si particles determined using BET N₂ sorption. *p<0.05 when compared with each other.

3.4.2 Effect of Silica Particle on H₂O₂ Decomposition

100 μM of H₂O₂ was mixed with each type of SiNP, and the change in the concentration of H₂O₂ was evaluated using FOX assay (**Figure 11**). The concentration of H₂O₂ is set to mimic the H₂O₂ concentration in skin tissue after an injury (50-100 μM)¹⁰⁶. When treated with NSi, the relative concentration of H₂O₂ decreased slowly and over 80% of the initial amount of H₂O₂ remained even after 72 hrs of incubation. Increasing particle porosity and acid treatment further decreased H₂O₂ concentrations (62.26±1.05 and 35.12±4.05 μM at 72 hrs for HSi and AHSi, respectively). The accelerated degradation of H₂O₂ resulted from a combination of H₂O₂-Si-OH complexation on the surface and mild catalytic effect of silica on H₂O₂ degradation^{44, 101}. As a result, the increase in surface

area of the silica particle through increased porosity combined with the change in surface chemistry led to a faster reduction in H_2O_2 concentration. However, these SiNP were not as effective as that of catalase, which completely decomposed H_2O_2 within 1 hr.

Figure 11. Relative H_2O_2 concentration after incubation with Si particles or 1000 units of catalase at pH 7.4 and 37°C . The starting H_2O_2 concentration was $100\ \mu\text{M}$.

3.4.3 Preparation and Characterization of Composite Adhesives

Catechol moieties were oxidized by NaIO_4 , resulting in the crosslinking and polymerization of catechol. PEG-DA is end-modified with catechol and oxidative crosslinking of catechol resulted in the curing of PEG-DA. The gelation time for PEG-DA averaged around 36 s (**Figure 12**), when the precursor solution mixture stopped flowing in a tilted vial. Si particles were incorporated into PEG-DA to form composite

adhesives. Incorporating NSi reduced the gelation time slightly (31 ± 0.82 s for PEG-NSi). The PEG network likely formed physical entanglement with SiNP and catechol moiety can form hydrogen bonds with Si-OH. These interactions contributed to the reduction in gelation time as a result of forming new crosslinking points within the adhesive network. Incorporating porous Si particles further reduced the gelation time (22 ± 0.82 s and 21 ± 0.82 s for PEG-HSi and PEG-AHSi, respectively). Increased particle surface area likely increased the number of interactions between catechol and the particle surface, effectively increased the rate of gelation.

Figure 12. Gelation time of PEG-DA and its composite containing different silica particles. * $p<0.05$ when compared to PEG-DA. ** $p<0.05$ when compared to PEG-DA and PEG-NSi.

ATR-FTIR spectra of the Si particles revealed Si–O–Si peaks at 1065 cm^{-1} (Si–O stretching) and 800 cm^{-1} (Si–O bending) as well as Si–OH bending peak at 950 cm^{-1} (**Figure 13**). The Si–OH peak intensity was noticeably increased for AHSi, which is in agreement with the EDS result. The spectrum of PEG-DA exhibited characteristic peaks corresponding to PEG (–C–O–C– at $1000\text{--}1150\text{ cm}^{-1}$, $\text{–CH}_2\text{–}$ at 2880 cm^{-1}), carbonyl group in ester bond (1729 cm^{-1}) and aromatic ring in catechol ($1400\text{--}1500\text{ cm}^{-1}$)⁶⁴. Incorporating the Si particles resulted in shifts over the peaks associated with Si–O–Si (1030 cm^{-1} and 790 cm^{-1}) and Si–OH (960 cm^{-1}), confirming the presence of Si particles in the composite adhesives. The shifts may be attributed to the overlapped signal from PEG ether peaks (–C–O–C– at $1000\text{--}1150\text{ cm}^{-1}$)¹⁰⁷. Additionally, the peaks associated with silica particle demonstrated reductions in intensity which indicated hydrogen bond formed between the Si–OH and catechol or the oxygen atom of –C–O–C– of PEG backbone.

Oscillatory rheometry was utilized to assess the effect of Si particles on the viscoelastic properties of the adhesives (**Figure 14**). For all adhesive formulations, the storage modulus (G') values exceeded those of the loss modulus (G''), indicating that these adhesive were chemically crosslinked¹⁰⁸. G' values for PEG-DA at the linear viscoelastic range were measured to be approximately 4.9 kPa, and adding 10 wt% NSi particles significantly increased G' (5.5 kPa for PEG-Si; $p < 0.05$ when compared PEG-DA). This increase in the stiffness of the adhesive network is potentially due to increased interactions between the Si particles and PEG-DA network^{64, 86}. Additionally,

incorporation porous Si particles further increased G' values (5.9 and 6.2 kPa for PEG-HSi and PEG-AHSi, respectively; $p < 0.05$ when compared PEG-DA and PEG-NSi).

Figure 13. FTIR spectra of the Si particles (A) and PEG-DA containing the Si particles (B). Dash lines indicate (from left to right for A and B) Si-O-Si (1065 and 1030 cm^{-1}), Si-OH (950 and 960 cm^{-1}), and Si-O-Si (800 and 790 cm^{-1}) peaks.

Additionally, the decrease in the particle size and increase in the effective surface area also contributed to the increased stiffness of PEG-DA composites ⁵⁵. This suggests that increased particle surface area likely increased the number of interactions between

Figure 14. Storage (A) and loss (B) modulus of PEG-DA and its composite containing different silica particles.

catechol and the particle surface, effectively increased mechanical property of the composite adhesive. G'' values for SiNP-containing composite adhesive were significantly higher when compared to that of PEG-DA. This result indicates that incorporating SiNP increased viscous dissipation properties likely through the formation of physical interactions within the adhesive network ^{64, 86}.

Bovine pericardium was used as the test substrate in lap shear adhesion test (**Figures 15**). The adhesive strength for PEG-DA was measured to be 3.84 ± 0.60 kPa. While it is difficult to compare reported values from other PEG-catechol adhesives due to differences in the method and substrate use, the values reported here are well within the range of 103-104 Pa which is similar to the storage modulus reported elsewhere for PEG-catechol adhesives ^{64, 86}. This value increased with the addition of 10 wt% SiNP (4.97 ± 1.00 , 6.87 ± 1.35 , and 7.35 ± 1.01 kPa for NSi, HSi, and AHSi, respectively). The addition of nanoparticles has been previously found to increase the adhesion strength in catechol-based adhesives ^{64, 86}. Measured lap shear adhesion strength for PEG-AHSi was the highest. The increased surface area in these particles likely increased the availability of Si that can interact with catechol found in PEG-DA for forming hydrogen bonding. This resulted in an increased bulk mechanical property and subsequently the adhesive property. Similarly, elevated bulk mechanical property for PEG-HSi and PEG-AHSi (**Figure 14**) also contributed to enhanced measured lap shear strength.

Figure 15. Lap shear adhesion strength of PEG-DA and its composite containing different silica particles using bovine pericardium as the test substrate. * $p < 0.05$ when compared to PEG-DA. ** $p < 0.05$ when compared to PEG-DA and PEG-NSi

In vitro degradation of the adhesive was studied by tracking the change in the dry mass of the fully crosslinked samples (**Figure 16**). PEG-DA degraded completely by week 8, which is in agreement with PEG-catechol adhesive with ester linkages⁶⁴. Incorporating Si particles delayed the degradation of the composite adhesives. For PEG-AHSi, approximately 40% of its original dry mass remained even after 8 weeks of degradation, and the degradation rate was found to be approximately 8% per week. The delayed degradation is likely a result of an increase in mechanical integrity as well as localized pH reduction surrounding the silica particle^{64, 86}. As amorphous silica particle degrades, soluble silica species in the form of orthosilicic acid is released¹⁰⁹, which contributed to

the delayed degradation of SiNP-containing adhesive. PEG-DA degrades through the hydrolysis of the ester linkage between the glutaric acid and PEG ¹¹⁰, and the hydrolysis rate of ester bond is reduced in a mildly acidic pH ¹¹¹.

ICP-OES was used to analyze the concentration of soluble Si released from Si particles (**Figure 17**). Soluble Si can be derived from hydroxylated silicon surface in the form of orthosilicic acid. PEG-NSi and PEG-HSi released less than 10 mg/L of Si over 24 hours, whereas PEG-AHSi released nearly 3 folds higher Si over the same period of time. This increased level of soluble Si suggests that acid treatment altered the surface chemistry of Si particle to form the more hydrophilic Si-OH functional group. We speculated that the nature of our particle synthesis resulted in noncrystalline particles which can react promptly to form surface groups of Si-OH when being exposed to oxidizing species or, in some cases, deionized water ¹¹². Additionally, the degradation rate of the silica particle was reported to be highly dependent on surface functional groups including SiOH, their protonated form (SiOH^{2+}), and their deprotonated form (SiO^-) ¹¹³. As a result, the increases in degradation products, soluble silica or silicic acid, were expected as the SiOH surface functional groups were increased after the acid treatment as demonstrated by the EDS results.

Figure 16. Normalized residual weight of PEG-DA and its composite containing different silica particles. The samples were incubated in PBS (pH 7.4) at 37 °C. * $p < 0.05$ when compared to all other groups

PEG-DA continuously generated H_2O_2 and reaching a maximum concentration of nearly 60 μM 6 hours after mixing the precursor solutions (**Figure 18**). Although PEG-DA cured around 36 s (**Figure 12**), catechol can continue to polymerize to form oligomers consisted of 6-7 catechol residues and generate H_2O_2 during catechol oxidative crosslinking^{40, 85}. The oxidation of catechol produced superoxide which can participate to further oxidize other catechol groups. The generated superoxide can further oxidize catechol or interact with protons (H^+) in the surrounding media to form H_2O_2 . After catechol ceased to undergo oxidative crosslinking, PEG-DA ceased to generate H_2O_2 , and the amount of H_2O_2 released from the adhesive decreased⁴⁰. Adding SiNP had minimal

Figure 17. Soluble Si content of PEG-DA and its composite containing different Si particles after 24 hour incubation in complete medium at pH 7.4 and 37°C. * $p < 0.05$ when compared to all other groups

effect on the measured H_2O_2 concentration. However, adding the porous HSi and acid-treated AHSi drastically reduced the amount of the released H_2O_2 ($44.27 \pm 1.50 \mu M$ and $34.30 \pm 1.83 \mu M$ for PEG-HSi and PEG-AHSi, respectively, at 6-hour time point). This result indicated that incorporating Si particles with enhanced surface area and surface modification can effectively reduce the amount of H_2O_2 generated during catechol crosslinking.

Figure 18. H_2O_2 concentration of PEG-DA and its composite containing different Si particles. * $p < 0.05$ when compared to all other groups. ** $p < 0.05$ when compared to PEG-DA and PEG-NSi

3.4.4 Effect of Incorporating Si Particle in PEG-DA on Cellular Response

The effect of incorporating Si particles into PEG-DA on the cell viability and proliferation of primary fibroblast, keratinocyte, and tenocyte was investigated. For fibroblast and tenocyte, PEG-DA reduced the relative cell viability to around 85% when compared to the media control (**Figure 19**), and the incorporation of AHSi particle significantly improves cell viability ($> 95\%$). This is presumably due to the reduced concentration of H_2O_2 released from PEG-AHSi. Interestingly, there was no difference in the cell viability for keratinocyte when exposed to the three different adhesive

formulations. Keratinocytes are more resistant to oxidative stress and can actively degrade ROS¹¹⁴. Keratinocyte viability was high even when it was exposed to PEG-DA, which generated the highest concentration of H₂O₂ among the adhesive formulation tested. An MTT assay was used to determine cell viability and normalized by cells that were exposed to the cell culture medium. The result demonstrated that keratinocytes, which were exposed to the adhesive extracts, had higher relative MTT signal when compared to control. This increase in relative MTT signal was not observed in RDF and HT. Specifically, keratinocytes exposed to the 3 adhesive formulations exhibited 30% higher MTT absorption than the control cells, indicating that exposure to H₂O₂ likely increases keratinocyte proliferation¹¹⁴.

Ki-67 expression was utilized to determine the number of proliferating cells after exposure to the adhesive formulation (**Figure 20**). For all cell types tested, cells exposed to PEG-DA exhibited the lowest fraction of proliferating cells, which increased with the incorporation of porous Si particles. Cells exposed to PEG-AHSi exhibited the highest-level cell proliferation, a 3-4-fold increase when compared to cells exposed to PEG-DA. Among the particles fabricated, AHSi releases the highest amount of soluble Si (Figure 10), which contributed to the observed increase in cell proliferation^{76, 115}. The cellular proliferation effect was not localized (Figure S6), potentially due to the rapid diffusion of soluble Si in the culture¹¹⁶. Additionally, AHSi also significantly reduced the concentration of H₂O₂ generated by catechol, which effectively promoted cell survival.

Taken together, SiNP with increased porosity and modified surface chemistry was utilized to control H_2O_2 generated during the curing process of catechol-based adhesive. SiNP functioned as a filler in the composite adhesive and effectively increased its crosslinking density and materials property. Most interestingly, these particles and their composites were nearly chemically identical. However, the cellular response to these materials was drastically different. Specifically, tenocytes are typically exposed to an environment with low oxidative stress due to ischemic localized tissue compression in tendons as well as the lack of vascularization in the native tissue ^{117, 118}. Exposure to reduced concentrations of H_2O_2 increased the cell viability and proliferation of tenocyte. On the other hand, keratinocytes can actively degrade ROS, and H_2O_2 has been previously demonstrated to promote keratinocyte proliferation and migration ¹¹⁴. Keratinocyte demonstrated cell viability that was independent of the concentration of the released H_2O_2 . Our study points to the need to control H_2O_2 concentration depending on the desired application. Depending on the biological system, the response to the released H_2O_2 will likely be significantly different.

When compared to catalytic metal oxide and ions, SiNP provides a biocompatible alternative to decompose H_2O_2 . Although SiNP demonstrated reduced capability in degrading H_2O_2 when compared to catalase, SiNP reported here provided added benefits besides catalyzing the degradation of H_2O_2 . Degradation product of SiNP mainly consists of silicic acid, which are present in various tissues (e.g., bone and tendon) or organs (e.g., liver and kidney) ^{119, 120}. Silicic acid can also promote collagen synthesis and osteoblast differentiation in vitro ^{121, 122}. Soluble Si has also demonstrated the ability to promote cell

Figure 19. MTT signal relative to culture medium control for primary rat dermal fibroblast (**A**), human epidermal keratinocytes (**B**), and human tenocyte (**C**) after incubated with PEG-DA and its composite containing different Si particles for 24 hours in complete medium at pH 7.4 and 37°C. * $p < 0.05$ when compared to PEG-DA. ** $p < 0.05$ when compared to all other groups, respectively.

Figure 20. Percentage of cells that were Ki-67 positive and the total cell count for primary rat dermal fibroblast (A), human epidermal keratinocyte (B), and human tenocyte (C) exposed to adhesive for 24 hours in complete medium at pH 7.4 and 37°C. * $p < 0.05$ when compared to all other groups

proliferation and migration, and accelerate dermal wound closure^{76, 115}. PEG-AHSi released the highest amount of soluble Si, which contributed to the highest Ki-67 expression in all three cell types that we tested. The incorporation of surface-treated SiNP is potentially a facile and economical method to control H₂O₂ concentration while improving the biocompatibility and adhesive performance of a PEG-based adhesive. Additionally, by better controlling the concentration of H₂O₂ generated during the crosslinking process of catechol-based adhesive, it can potentially enable these adhesives for cell-engineering or cell delivery applications involving cells that are sensitive to elevated oxidative stress.

3.5 Conclusions

In conclusion, increased porosity and acid treatment of SiNP greatly enhanced its ability to decompose H₂O₂. When these SiNP were incorporated into PEG-DA, the amount of released H₂O₂ was also significantly reduced. Additionally, incorporating these particles increased lap shear adhesion strength, storage modulus, and altered the adhesive degradation behavior. Furthermore, these SiNP increased the cell viability of primary dermal fibroblasts and tenocytes, and the proliferation rate of these cells and keratinocytes. Ultimately, incorporation of porous and acid-treated SiNP can be a useful approach to simultaneously modulate the concentration of H₂O₂ while increasing the adhesive performance of catechol-based adhesives.

3.6 Acknowledgements

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4 Systemic Study of Silica Particle-Catechol Composite Adhesive on Dermal Wound Repair in Mice³

4.1 Abstract

The robust optimum design of the experiment was utilized to pinpoint catechol-based polyethylene glycol (PEG) adhesive and their composite for the dermal wound healing specific application. Experimental results confirmed that the incorporation of an acid-treated silica particle (AHSi) could reduce hydrogen peroxide byproduct of the catechol-based adhesive in a predictable range. The full-thickness dermal wound repair model in mice was performed to study the effect of polymer architecture and incorporation of AHSi. 6-arm PEG adhesive (PEG-D6) with the H_2O_2 producing nearly 100 μM was shown to shorten the time required for wound closure by half. The addition of AHSi was found to improve the tissue remodeling while maintaining the effective wound closure speed observed in the PEG-D6. Ultimate, tissue-specific adhesive architecture and composite formulation can be design to achieve the desired outcome, combing accelerated wound healing with better wound remodeling.

4.2 Introduction

Wound healing is one of the most commonly studied topics in biomedicine. A number of approaches have been proposed over the decades of biomedical material development to treat tissue-specific wound healing. These approaches may include scaffolding, drug and

protein delivery, tissue engineering and transplantation, and bioactive dressing. Wound dressing required means to adhere to skin or target tissue to provide structural support or to cover the wound entirely. However, there are challenges when dealing with an open wound or topical wound, which involves fluid management, wound irrigation, antimicrobial, and sufficient material strength. Particularly, the most wound dressing will not perform well in a wet environment ¹²³⁻¹²⁵.

Catecholic amino acid, 3,4-dihydroxyphenylalanine (DOPA), are naturally contained within mussels adhesive proteins, which enables them to bind underwater surfaces ^{123, 126}. While oxidized, DOPA is capable of forming interfacial covalent bonds with organic surfaces (e.g., tissues), making an interesting candidate for wound dressing under wet environment ^{127, 128}. Catechol can be functionalized into synthetic polymers (i.e., polyethylene glycol) to produce strong, wet adhesive properties for various applications ¹²⁹⁻¹³¹.

Catechol adhesive must undergo oxidation in order to crosslink and adhere to surfaces ¹³²⁻¹³⁵. However, while doing so, the adhesive was found to release chemical byproduct in the form of superoxide and hydrogen peroxide (H_2O_2) ^{36, 40, 136}. The amount of superoxide and H_2O_2 was measured to be between $10-10^4 \mu M$ range, depending on their architecture ³⁶. Recently, we have demonstrated that the incorporation of an acid-treated silica particle (AHSi) can reduce H_2O_2 concentration from a polyethylene glycol-based catechol adhesive. The composite adhesive demonstrates improved stiffness, adhesion strength, and improved cellular response in vitro. The biological response was investigated with

fibroblast, keratinocyte, and tenocyte¹³⁶. These cell types are known to react to H₂O₂ and oxidative stress differently. Keratinocyte is known to demonstrate high resilience toward H₂O₂ and oxidative stress following by moderately H₂O₂-tolerate dermal fibroblast and non-H₂O₂ tolerant tenocyte^{35, 40, 118}. Hence, the ability to modulate H₂O₂ concentration is required for the optimum biological response when targeting such cells and tissues.

To tune the H₂O₂ concentration of the adhesive, we introduce a bioactive silica particle, AHSi, into the adhesive network. The architecture of the adhesive network (i.e., different branch structure, crosslinking density, etc.) is known to inherently affect the H₂O₂ concentration^{40, 136}. We have demonstrated previously that the incorporation of AHSi particle effectively reduces H₂O₂ concentration by 50% in an 8-arm polyethylene glycol (PEG) based adhesive, potentially due to the surface interaction between silanol group on the particle surface with H₂O₂ under wet condition¹³⁶. We hypothesized that variation of PEG adhesive architecture would allow for better control over H₂O₂ generation while maintaining the adhesive properties of the composite. The resulting composite adhesive should also demonstrate a better biological response as a result.

To this end, we systematically study the varying catechol adhesive composite containing AHSi. Gelation time, H₂O₂ concentration, and adhesive strength of the adhesive and their composites have been investigated. Full-thickness dermal wound repair mice model study was carried out to determine the effect of different adhesive architecture as well as the effect of AHSi addition on dermal wound healing.

4.3 Materials and methods

4.3.1 Materials

4-arm, 6-arm, and 8-arm Polyethylene glycol (10kDa, 15 kDa, and 20 kDa, respectively, Jenkem, TX, USA), Sodium periodate (NaIO_4 , >99.8%) were obtained from Acros Organics (Fair Lawn, NJ). Phosphate buffer saline (PBS), tetraethyl orthosilicate (TEOS, 99.8%), ethanol (200 proof), sodium hydroxide, and acetic acid (Glacier) were purchased from Fisher Scientific Co. (Pittsburgh, PA). Histology mounting medium Polyfreeze, Trichrome Stain (Masson) Kit, Bouin's solution, and Weigert's iron hematoxylin solution were purchased from Sigma-Aldrich (St. Louis, MO). goat anti-rabbit IgG H&L (Alexa Fluor 488) (ab150077), anti-CD68 antibody (ab125212), and goat anti-rabbit IgG H&L (Alexa Fluor 647) were purchased from Abcam (Cambridge, MA). 4,6-Diamidino-2-phenylindole (DAPI) was obtained from Invitrogen (Grand Island, NY).

4.3.2 Synthesis, Preparation, and Characterization of PEG-DA Adhesives and their composites

The synthesis of PEG-DA adhesive was synthesized with 4, 6, and 8-armed PEG (MW=10, 15, and 20 kDa, respectively, JenKem USA, TX) according to a previously published protocol ⁶⁴. After purification, the adhesive was analyzed via NMR (Varion 400 MHz) to determine the catechol coupling efficiency via ^1H -NMR with D_2O .

The preparation of PEG-DA adhesive was done according to a previously published protocol¹³⁶. Briefly, PEG-DA (100, 225, or 300 mg) was dissolved in 1 ml of PBS solution (1x, 2x, or 4x) to create a PEG-DA precursor solution. The addition of acid-treated silica particle (AHSi) (0, 5, or 10%wt) was done in the precursor solution to create a composite precursor solution. The particle was synthesized previously with the published protocol¹³⁶. 11.6 mg of NaIO₄ was dissolved in 1 ml of deionized water to create NaIO₄ solution. The precursor solution and NaIO₄ solution were mixed at a 1:1 v/v ratio to create PEG-DA adhesive and composites. The gelation time of the adhesive and the composite was determined with vial tilting technique as the adhesive gel rapidly. The adhesive was considered gelled when the solution ceased to flow in a scintillated vial.

Lap shear adhesion test was performed using bovine pericardium while following a previously published protocol according to ASTM standard F2255-05⁶⁴. Strips of pericardium tissues (2.5 cm x 2.5 cm) were kept moist in PBS. 100 µl of PEG-DA precursor solution and NaIO₄ solution with and without Si particles were mixed in a scintillated glass vial before being transferred on to the surface of the tissue. Another piece of pericardium tissue was applied over the adhesive to create an adhesive joint (overlapping area of 2.5 cm²), which was compressed with a 100-g weight for 15 min. The adhesive joint was submerged in PBS (pH = 7.4) at 37 °C overnight prior to testing. A digital caliper was used to measure the dimensions of the overlapped area of each adhesive joint before testing. The fixation of the tissue pieces on to the test stage was done with serrated metal grips to ensure sufficient traction. An Electroforce® machine (Bose Electroforce Group, MN) was used to pull apart the adhesive joint to failure at a

rate of 0.1 mm s⁻¹. The adhesive strength was determined by the maximum load divided by the initial overlapped area of the adhesive joint.

4.3.3 Systemic Study of H₂O₂ Release in PEG Adhesive Composites

The robust design was utilized to predict the H₂O₂ concentration. The prediction was made utilizing the measured data from multiple experiments. Each factor that affects the material behavior was listed and ranked according to how large the effect of their change can be. PEG architecture, PEG precursor concentration, PBS concentration, and amount of incorporated AHSi were identified as largely affect the properties of the adhesive. The three properties that would significantly affect the material performance were determined (including gelation time, H₂O₂ concentration, and adhesive strength) as they are the most commonly reported value ¹³⁶. Each factor was calculated through the mean value retrieved from real experiments. The mean observation value of the experiment was used to calculate the signal to noise ratio. Mean signal to noise ratio was then used to assign the weight to the factor effects. The equation used to find the signal to noise ratio for each formulation is as follow:

$$S/N = -10 \times \text{LOG}(\text{MSO}) \quad (4)$$

Where S/N is signal to noise ratio and MSO is mean squared observation. MSO can be calculated by

$$\text{MSO} = ((1 \div X_1^2) + (1 \div X_2^2) + \dots + (1 \div X_n^2)) / n \quad (5)$$

Where X is the experimental data of each repeat, subscript denotes the repeat counts, and n is equal to the total number of repeats.

The factor effect can then be compiled from compiling signal to noise ratio into a 3*4 matrix assigning the signal to noise prediction to each factor to each set of change ^{137, 138}. Nine sets of experiments were conducted to collect the data needed for the predictions (Table 4).

Table 4. Formulation required to fulfill the robust design matrix requirement

Experiment	Factors			
	PEG Architecture	PEG conc. (mg/ml)	PBS conc. (x0.01M)	%wt AHSi
1	4	150	1	0
2	4	225	2	5
3	4	300	4	10
4	6	150	2	10
5	6	225	4	0
6	6	300	1	5
7	8	150	4	5

8	8	225	1	10
9	8	300	2	0

4.3.4 Systemic Study of PEG Composites in Dermal Wound Model in Mice

The adhesive formation was selected to minimize the number of mice required to obtain meaningful data from the animal experiment. The first study criterion is the effect of the particle incorporation, which is a continued investigation from the in vitro result previously published from our group ¹³⁶. The formulations were chosen to demonstrate the effect of the particle on the predicted reduction in H₂O₂ concentration and the increase in soluble silica concentration released from the composite. The second study criterion is the effect of the PEG-DA structure and their native H₂O₂ byproduct. The formulations were chosen to demonstrate the effect of the architectural alteration as well as the change in H₂O₂ concentration and release profile (**Table 5**). The number of mice required for bilateral experiment with three extra mice as backup is equal to 17 (**Table 6**)

Table 5. Formulation to be tested with the full thickness dermal wound in mice

Formulation	Silica Composite	Group
Control	None	A
PEG-D6	None	B
PEG-D4	10%wt AHSi	C

PEG-D6	10%wt AHSi	D
PEG-D8	10wt% AHSi	E

Table 6. Full list of treatments for all mice in the full thickness dermal wound repair experiment

Mice#	L	R	Time (Day)
1	A	B	14
2	B	C	14
3	C	D	14
4	D	E	14
5	E	A	14
6	B	A	14
7	C	E	14
8	D	C	14
9	B	E	7

10	A	E	7
11	A	D	7
12	E	D	7
13	C	A	7
14	D	B	7
15	E	C	7
16	B	D	7
17	C	B	7

4.3.5 Full Thickness Dermal Wound Repair model

Female wild type mice (age 9-10 weeks, weight 20 g) were used to study the full-thickness dermal wound model. All mice were subjected to a full-thickness wound model protocol modified from previously published protocols (**Figure 21**)¹³⁹⁻¹⁴¹. Mice were anesthetized with isoflurane before the hair was removed with electric shear and hair removal cream. One day after hair removal, the mice were anesthetized, and a 5 mm

Bandages Dimension:

Adaptic Mesh: 1.2-1.5 cm * 3 cm

First Hydrofilm Bandage: 2.5-3 cm* 5 cm

Second Hydrofilm Bandage: 3.5-4 cm*15 cm

Figure 21. Illustration (A) and photograph (B-I) of the step-by-step process of full-thickness wound repair model in mice.

tissue punch was used to create the wound on the back of the mice bilaterally to ensure identical wound size. Medical-grade silicon ring (outer diameter 10 mm, inner diameter 6 mm) was fixated to the wound as a splint to prevent skin movement and change in wound size due to skin relaxation and shrinkage. The ring was first fixed with cyanoacrylate glue

then 5-0 nylon suture was used to fix the ring with the skin. Four suture spots were used to ensure tight fixation as the fixing ring must survive until day 7 or 14. The wound was left open or was treated with PEG-DA adhesive and their composites. Images of the wound were taken for wound size analysis. The adhesive was allowed to gel for 2 minutes before the wounds are covered with a non-stick wound dressing mesh (Adaptic). Sticky and breathable adhesive film (Hydrofilm) was used to attach the non-stick mesh on to the skin surrounding the wound. A large adhesive film piece was then used to completely seal off the wound from the surrounding element. The pain killer was given to the mice at 20 μ l/day for three days after the surgery to ensure animal comfort. Wound dressing was inspected and replaced every day for the first four days after surgery. Images were taken at the same time as the wound redressing. Depending on the time group the mice were in, they were euthanized on day 7 or 14.

4.3.6 Immunological and Histological Analysis of Dermal Wound Model

After 7 or 14 days, the animals were euthanized via CO₂ asphyxiation. The images of the extracted tissue samples were taken for wound size analysis via an Olympus microscope. The tissue samples were fixed in Polyfreeze and flash-frozen in a liquid nitrogen bath. The tissue was kept in -80°C for up to 4 weeks before sectioning with a cryomicrotome. The tissue slices were processed at the thickness of 10 μ m mounted onto Histobond slide. There were 3 tissue section on each slides, at least 10 mounted tissue slides were produced for each tissue sample.

The immunological analysis of the dermal wound was done via CD68-DAPI fluorescence staining for macrophage population. The macrophage staining was done according to previously published protocol with minor modification ⁶⁴. Samples were obtained from the -80°C freezer and fixed in 100% ethanol for 2 minutes. Samples were then washed 3 times in PBS with Tween 20 (PBST) - 5 minutes each wash. Circles were then drawn around each sample using a hydrophobic marker. Endogenous blocking was completed using 10% Goat Serum diluted using 1% Bovine Serum Albumin (BSA) for 60 minutes. Samples were washed again in PBST 3 times - 5 minutes per wash. Next, samples were incubated in a 1/100 dilution Primary Anti-CD68 Ab (ab125212 – Rabbit Polyclonal) for 12-14 hours at 4°C in a humidified chamber. Samples were washed again in PBST 3 times - 5 minutes per wash and the lights remained off for the remainder of the stain. The samples were then incubated in a 1/200 dilution of the Secondary Antibody (Goat Anti-Rabbit IgG H&L – AlexaFluor488) for 60 minutes at room temperature. Samples were washed again in PBST 3 times - 5 minutes per wash before being incubated in a DAPI antibody (1/1000 dilution) for 3 minutes and then the wash was repeated. Samples were mounted using an aqueous mounting medium and were imaged with 2-3 hours of the stain being completed. CD163 (M2-polarized macrophage stain) was not used. Image of the sample was taken immediately after the staining.

The histological analysis of the dermal wound tissue was done via trichrome staining for wound morphology, epidermis analysis, and collagen content analysis. The trichrome staining was done according to previously published protocol ⁶⁴. Samples were mounted using a permanent mounting medium. Additionally, keratin 6 staining was utilized to

analyze the wound maturity through the population of keratinocyte. The keratin 6 staining was done according to previously published protocol with minor modification¹⁴². Instead of counterstaining with toluidine blue to stain the nuclei, Gill's 3 hematoxylin solution (Sigma Aldrich) has been used in place of toluidine blue. Briefly, samples were rinsed in tap water and then immersed into the hematoxylin solution for 1-5 minutes. Samples were then rinsed in running tap water until the water was colorless. The samples were then dipped 10 times in an acid rinse solution (2mL glacial acetic acid + 98mL diH₂O) followed by 10 dips in cool tap water. Then slides were dipped 5 times in a bluing solution (0.3mL NH₄OH + 100mL tap water) followed by 20 dips in tap water. Samples were then mounted using a permanent mounting medium and were stored overnight at 4°C before imaging.

Trichrome and keratin 6 stained tissue samples were imaged via EVOS microscope under polarized light. The overlaid images were processed via the Adobe Photoshop program auto stitching module. All image was measured and analyzed via ImageJ with wound healing tool macro with modifications¹⁴³.

4.3.7 Statistical Analysis

Statistical analysis was performed using JMP Pro 13 application (SAS Institute, NC). One-way analysis of variance (ANOVA) with Tukey-Kramer HSD analysis was performed for comparing means. $p < 0.05$ was considered significant.

4.4 Results and discussion

4.4.1 A systemic study of H₂O₂ release in PEG adhesive composites

The PEG-D4, PEG-D6, and PEG-D8 adhesive were synthesized (**Figure 22**). NMR spectra of each PEG adhesive have been analyzed (Appendix A). The coupling efficiency of each PEG adhesive was found to be over 90%. Ensuring their performance as adhesives with catechol functionalization. The formulation tested can be seen in **Table 7**. The difference between the predicted value and the experimental data are apparent on the effect of incorporating silica particle into the adhesive (**Figure 23A and C**). Adding silica particle was proven to reduce gelation time and increase adhesive strength^{86, 136}. However, the prediction model does not agree with this understanding. On the other hand, prediction on H₂O₂ concentration is effective enough to be able to predict the concentration of H₂O₂ at a 10-20% increase from the tested value (**Figure 23B**). Interestingly, the addition of AHSi provided a reduction in H₂O₂ by 20.24±0.67 µM regardless of PEG architecture, PEG concentration, and PBS concentration. The clearly limited reduction indicated that the effect of the particle on H₂O₂ reduction is governed by the available binding site on the particle^{45, 136}. As a result, the prediction model is not perfectly predicted the H₂O₂ concentration and completely ineffective in predicting gelation time. However, the prediction on the trend of H₂O₂ can be utilized to narrow down the formulation to be tested via maximum H₂O₂ concentration. The predicted formulation that fits the criteria for dermal wound repair can be seen in **Table 8**. All prediction data can be seen in **Appendix A**.

Figure 22. Chemical Structure of PEG-D4, PEG-D6, and PEG-D8. Single carbon (A), dipentaerythritol (B), and hexaglycerol (C) were used as the center group.

Table 7. Experimental result of the formulations required to fulfill the robust design matrix requirement

Experiment	Gelation Time (second)	H ₂ O ₂ (μM)	Adhesive Strength (kPa)
1	273.33±8.98	92.35±2.01	-
2	45.83±3.44	89.30±1.72	3.17±0.18
3	167.50±4.79	68.31±2.14	3.62±0.29
4	515.00±13.84	52.36±1.91	-
5	39.17±1.68	78.17±2.08	3.10±0.20
6	52.83±2.27	72.22±2.15	5.31±0.16
7	358.33±7.45	26.85±2.59	-
8	85.50±4.23	32.33±1.49	4.26±0.29
9	37.50±4.79	61.35±3.01	7.07±0.63

Table 8. Predicted formulation that fit the criteria for dermal wound repair

PEG Architecture	Concentration (mg/ml)	PBS conc.	%wt AHsi	Gelation Time	H₂O₂ conc.	Adhesive Strength
6	300	2	0	35.94	108.84	5.74
6	300	2	10	95.57	69.54	5.11
8	300	2	10	97.24	39.04	6.23
4	300	2	10	119.58	86.27	4.52

Figure 23. Prediction vs. Experimental result of gelation time (A), maximum H₂O₂ concentration (B), and adhesion strength (C). *p<0.05 when compared to their pair

4.4.2 Dermal Wound Model in mice

4.4.2.1 Wound closure

Wound size measurement on the mice was done in a time-dependent manner. There was no difference in the wound size immediately after the surgery (**Figure 24** and **25**). However, after 7 days, the size of all the adhesive treated wounds is significantly smaller than the control wound. This is potentially due to the introduction of a physical coverage

Figure 24. Representative image of the harvested tissue at day 0 (top), 7 (middle), and 14 (bottom) of control (A,F,K), PEG-D6 (B,G,L), PEG-D6-AHSi(C,H,M), PEG-D4-AHSi (D,I,N), and PEG-D8-AHSi (E,J,O). Red and yellow dash line indicates the wound area. Scale bars are 1 cm.

over the wound as well as the release of H_2O_2 in the first few days can help recruit immune cells as well as act as a chemotaxis source for keratinocyte recruitment. PEG-D8-AHSi was found to have the smallest wound size among all the adhesive treated wounds. This may be due to the fact that the composite releases the least amount of H_2O_2 , which might contribute to the shrinkage of the wound. Additionally, the wound treated with adhesives are seemingly completely closed.

On day 14, control wounds have the scar tissue three times larger than all of the adhesive treated wounds. All catechol-AHSi composite treated wounds were found to have reduced wound size when compared to control suggesting that incorporation of soluble silica can induced faster wound closure when compare to the open wound. Interestingly,

Figure 25. The wound area of the control wound and wound treated with different formulations of the adhesive. * $p < 0.05$ when compared to their pair

only PEG-D6 AHSi and PEG-D4 AHSi showed significant differences when compare to PEG-D6. We speculated that the H₂O₂ concentration level (50-80μM) and, presumably, increases in matrix stiffness contributed to such change when combined with the release of soluble silica^{40, 64}.

4.4.3 Immunological Analysis of Dermal Wound Model

To evaluate the effect of the particles on the biological response of the composite adhesives, catechol-based adhesive and their 10% wt AHSi composite with either 4,6, or 8 arms architecture were introduced in on to the dermal wound repair model for 7 and 14 days. 1*1 mm box was used to randomly selected the analysis area where the CD68 positive cells, labeled in both green and blue, was count against the total population of cells, labeled in blue. A total of 3 counts was done for each image within the wound proximity then the counts were calculated for the approximation of CD68 population ratio. The ratio of CD68 to total cell number was found to be the highest in the first 7 days then reduced to the lowest level in the control wounds (**Figure 26 and 28**). Interesting, both PEG-D6 and PEG-D6-AHSi demonstrated a significant reduction of CD68 population when compare to control on day 7 indicating lowered immunological response. Inversely, at day 14, the ratio of CD68 population of all adhesive and composite treated samples were found to be significantly higher than the control indicating increased immunological response (**Figure 27 and 28**). This increase in CD68 population on day 14 suggested a prolonged immunological response. However, the macrophages involving in wound healing were thought to be roughly divided into two

types, M1 – inflammatory response and M2 – matrix remodeling. M2 macrophages are responsible for suppressing inflammatory responses, matrix remodeling, and tissue regeneration ⁶⁴. The CD68 staining indicates only the population of macrophage without distinguishing the types of the cells. We speculated, with support from trichrome and keratin-6 results (**Figure 31** and **34**, respectively), that the wound remodeling macrophage is the dominant macrophage types in these wounds. The ratio of CD68 population was not significantly different between PEG-D6 and PEG-D6-AHSi indicating that the inflammatory response of the two treatment groups are similar. However, the differences between PEG-D4 and PEG-D8 to PEG-D6 and PEG-D6-AHSi were observed. These results indicating that the wound react to the release of soluble silica and narrow shifts in H₂O₂ as PEG-D4 has higher H₂O₂ than PEG-D6 while PEG-D8 has lower H₂O₂ concentration than PEG-D6. We theorized that the combination of soluble silica ($\geq 10 \mu\text{M}$) and H₂O₂ level at around 60 μM might be the ideal concentration to accelerate wound healing without causing significant increase in immunological responses similar to previous findings ^{64, 115}.

Figure 26. Representative CD68-DAPI immunological image of the harvested tissue on day 7 after treatment with control (A), PEG-D6 (B), PEG-D6-AHSi(C), PEG-D4-AHSi (D), and PEG-D8-AHSi (E). Yellow dash line box giving the example for the area used to calculate CD68 positive cells. White dash line indicates the wound proximities. Green = CD68 positive cells and blue = cell nucleus. Scale bars are 1 mm.

Figure 27. Representative CD68-DAPI immunological image of the harvested tissue on day 14 after treatment with control (A), PEG-D6 (B), PEG-D6-AHSi(C), PEG-D4-AHSi (D), and PEG-D8-AHSi (E). Yellow dash line box giving the example for the area used to calculate CD68 positive cells. White dash line indicates the wound proximities. Green = CD68 positive cells and blue = cell nucleus. Scale bars are 1 mm.

Figure 28. The ratio of CD68 positive cells of the wound treated with different formulation of the adhesive. * $p < 0.05$ when compared to their pair

4.4.4 Histological Analysis of Dermal Wound Model

Healthy skin tissue is comprised of the epidermis, dermis, panniculus muscle, and subcutaneous adipose tissue (**Figure 29**). When skin experience trauma, clotting cascade will be initiated to stop the bleeding, then an inflammatory response will start by recruiting neutrophil, macrophage, and fibroblast. The granulation tissue (gr), which is a vascularized, not-well-defined tissue, and connective tissue will start from. The amount of granulation tissue and connective tissue is governed by the healing process of the wound. With higher inflammatory response and heal poorly, higher connective tissue and collagen synthesis are to be expected. At a later stage of a healing wound, tissue

remodeling occurred, and the tissue structure will start to resemble a healthy skin structure. On the other hand, poorly healed scars will end up with an excess amount of collagen as well as poor tissue remodeling, causing the newly formed skin to look distinctly different than that of healthy skin.

Histological staining images were collected and analyzed with 2 tissue slides from 3 repeat of each sample group (n=6 for each group). Masson's trichrome staining is generally utilized to study the skin tissue for their morphology and structural content of

Figure 29. Histological analysis of skin structure and thickness. Scheme of skin structure (A), histological skin sections stained with Masson trichrome and collected from young (8-12 week-old) and old (16-20 week-old) mice fed with normal diet for a period of 8 weeks (B), quantification of the skin layers thickness: the epidermis (C) Epi - epidermis, dWAT - dermal white adipose tissue, F-rd fibroblast rich dermis; scale bar 200 μm. Adapted from reference ¹⁴⁴ under the terms of the Creative Commons Attribution License (CC BY 3.0)

the sample. Trichrome staining on skin tissue stains collagen in blue, muscles, and other connective tissues in red, and cell nucleus in dark brown (**Figure 29**).

The wound proximities were found to be in irregular shapes, which are the result of dermal contractions ¹⁴⁵. The results from tissue sample harvested on day 7 after surgery indicate a faster wound healing in the adhesive treated samples with thicker epidermis formation when compared to control (**Figure 30 and 32A**). This increased epidermis thickening or dermal hyperplasia is thought to be the effect of H₂O₂ treatment, as reported by others in the field ^{146, 147}. The thickest epidermis layer was observed in PEG-D4-AHSi, which has the highest H₂O₂ concentration among the composite samples following by PEG-D6-AHSi and PEG-D8-AHSi, which has the lowest H₂O₂ concentration (**Figure 23B**). PEG-D6 treated sample also demonstrated an increase in epidermal thickness, but the increase is not significantly different than those observed in PEG-D6 AHSi and PEG-D4-AHSi. The result suggested that the increase in epidermal thickness is not only affected by hydrogen peroxide concentration but also the release of soluble silica.

The collagen content determined through the measurement of collagen layer thickness. The thickness of the collagen layer in the control sample tissue is significantly thicker than those of the adhesive treated samples (**Figure 30 and 32B**). The granulation tissue formation was found to be the most prominent in PEG-D4-AHSi, which has the highest amount of H₂O₂ (>80 μ M) followed by PEG-D6-AHSi (between 40 and 80 μ M), and PEG-D8-AHSi (<40 μ M). Interestingly, PEG-D6 (approximately 100 μ M of H₂O₂)

treated wound demonstrate dermal layer structure, which resembles those of healthy tissue the most. This may be due to the fact that a high concentration of H_2O_2 (as low as 1000 μM for one-time dose) can lead to epidermolysis and rapid new epidermal formation with epidermal hyperplasia as evident in the epidermis analysis^{93, 147}. However, this result is not consistent across all the mice, which strongly suggested by the large standard deviation. This is to be expected as the lack of control over the release of H_2O_2 release as well as weaker material integrity, resulting in a large variety of biological responses.

Trichrome staining results of tissue samples harvested on day 14 after surgery indicate healthy wound remodeling on all adhesive treated samples (**Figure 31**). Smaller wound proximity, as well as increased granulation tissue and reduction in collagen layer thickness, have been observed in all adhesive treated formulation. PEG-D6 and PEG-D6-AHSi demonstrated tissue remodeling, which is resembling healthy tissue. A similar tissue remodeling trend was also observed in PEG-D4-AHSi, and PEG-D8-AHSi treated samples. However, the collagen thickness is higher in these groups when compared to the PEG-D6 and its composite. This might be attributed to the fact that the healing process of PEG-D6 involved early thermolysis and new epidermal formation since the hydrogen peroxide concentration of the adhesive is closer to 100 μM at their maximum concentration. For PEG-D6-AHSi, it is possible that the amount of sustained release of H_2O_2 resulted in a similar biological response at day 14 as the lagged healing process was observed in day 7 sample groups. PEG-D6—AHSi demonstrated the thinnest collagen layer (**Figure 32**) which might suggest that the wound remodel successfully at day 14.

Figure 30. Representative trichrome histological image of the harvested tissue on day 7 after treatment with control (A), PEG-D6 (B), PEG-D6-AHSi(C), PEG-D4-AHSi (D), and PEG-D8-AHSi (E). Black (A-B) and Yellow (C-E) dash line indicate the area used to calculate collagen content. White dash line indicates the wound proximities. es = eschar, gr = granulation tissue, and ep = epidermis. Scale bars are 1 mm.

Figure 31. Representative trichrome histological image of the harvested tissue on day 14 after treatment with control (A), PEG-D6 (B), PEG-D6-AHSi(C), PEG-D4-AHSi (D), and PEG-D8-AHSi (E). Black (A and C) and Yellow (B, D, and E) dash line indicate the area used to calculate collagen content. White dash line indicates the wound proximities. es = eschar, gr = granulation tissue, and ep = epidermis. Scale bars are 1 mm.

Figure 32. The epidermis thickness (A) and collagen layer thickness (B) of the wound treated with different formulation of the adhesive. * $p < 0.05$ when compared to their pair

Interestingly, all-composite adhesive treated samples demonstrated a significantly thinner epidermal when compare to PEG-D6 (approximately 25 μm vs. 140 μm), suggesting the

lack of epidermal hyperplasia. In fact, the thickness of the epidermal in these treatment groups matched with the thickness of epidermal in the healthy tissue in the wild-type and age-matched mice (**Figure 29C.**)¹⁴⁴. Interestingly, the keratinocyte population observed in both PEG-D6-AHSi and PEG-D8-AHSi (H_2O_2 concentration $<80\ \mu\text{M}$) are significantly higher than those observed in both PEG-D6 and PEG-D8-AHSi ($\text{H}_2\text{O}_2 >80\ \mu\text{M}$). The result suggested that AHSi addition combining with appropriate adhesive architecture or H_2O_2 concentration, i.e., PEG-D6-AHSi, can lead to wound remodeling, which resembles healthy tissue without epidermal hyperplasia.

Keratin-6 suggested that adhesive treatment increase keratinocyte recruitment. In addition, adding particle in the adhesive further increases keratinocyte recruitment on day 7 after surgery (**Figure 33 and 35**). Keratinocyte content is a sign of skin maturation as keratinocyte maturation completes the cornification of the skin, providing the outermost layer of protection to the dermal tissue below¹⁴⁸. The increase in keratin 6 positive cell population in the epidermis and surrounding tissue (approximately 82% with higher than 90% in the epidermis) in adhesive treated samples suggested that the skin can undergo maturation earlier than the control sample for at least a week. The population of keratinocyte was found to be concentrated in the epidermis and the surrounding tissue, which conforms with normal skin tissue structure^{149, 150}. H_2O_2 was found to increase keratinocyte proliferation as a response to oxidative stress^{38, 151}. Moreover, soluble silica is also reported to increase keratinocyte proliferation as well as induce fibroblast and keratinocyte migration^{76, 136}. Additionally, incorporation of silica particle increases the stiffness of the adhesive, which contribute to fibroblast and keratinocyte proliferation

Figure 33. Representative keratin-6 histological staining images of the harvested tissue at day 7 of control (A), PEG-D6 (B), PEG-D6-AHSi(C), PEG-D4-AHSi (D), and PEG-D8-AHSi (E). Red dash line indicates the area used to calculate keratinocyte content. White dash line indicates the wound proximities. Scale bars are 1 cm.

Figure 34. Representative keratin-6 histological staining images of the harvested tissue at day 14 of control (A), PEG-D6 (B), PEG-D6-AHSi(C), PEG-D4-AHSi (D), and PEG-D8-AHSi (E). Red and yellow dash line indicates the area used to calculate keratinocyte content. White dash line indicates the wound proximities. Scale bars are 1 cm.

Figure 35. The ratio of keratin-6 positive cells of the wound treated with different formulation of the adhesive. * $p < 0.05$ when compared to control in the same time group

when direct cell-material contact was established^{136, 152}. The control sample demonstrated the least amount of keratin-6 positive cells suggesting the lagged maturation progress.

Keratin 6 stainings on tissue samples harvested on day 14 after surgery showed an increase in keratinocyte population in control samples, while a general reduction in the population was observed in all samples with adhesive treatment. The keratinocyte population in all adhesive treatments were not significantly different and was found to be around 40% of the total cell population (approximately 40% with 90% in the epidermis). This reinforces the assumption that the introduction of adhesive treatment was contributing to the early maturation of keratinocyte and keratinocyte recruitment.

The population of keratinocyte reduced 14 days after surgery samples (**Figure 34** and **35**). This is presumably due to the early maturation of the keratinocyte allowing the

dermal tissue remodeling as keratinocyte are key cells that regulate dermal fibroblast activity and dermal wound healing ¹⁴⁹. This observation correlated with the trichrome staining result, which suggested the near-complete tissue remodeling in PEG-D6-AHSi treated sample (**Figure 31C**).

The combination of trichrome and keratin 6 (**Figures 32 and 35**) strongly suggested the H₂O₂ concentration-dependent response of dermal wound healing ^{147, 153}. The concentration of H₂O₂ that presumably accelerate wound healing was reported to be varying from 10-10³ μM. However, with the full thickness wound model result, we narrow down the concentration that specific to the dermal wound to be between approximately 40-100 μM. However, H₂O₂ alone does not contribute to fully remodeled tissue at 14 days after the injury, as suggested by epidermal hyperplasia observed in the PEG-DA adhesive treated samples (**Figure 31 and 32A**). The incorporation of AHSi, which can release soluble silica in a biologically relevant concentration, also contributes strongly to the epidermal formation and tissue remodeling ^{136, 148, 149}. The particle participates in reducing the H₂O₂ concentration while altering the material stiffness, which positively affects the wound healing process in vivo¹³⁶. The wound was remodeled to mostly resemble healthy tissue on day 14 after the injury, as demonstrated by trichrome staining. The lack of epidermal hyperplasia suggested no excessive thermolysis and inflammatory response took place during the healing process. AHSi or soluble silica was demonstrated to play a critical role in inducing keratinocyte and fibroblast proliferation and migration, which directly affect dermal wound healing in vitro ^{76, 136}. For the observed in vivo results, the author proposes the simplified relationship between

H₂O₂, material property, and AHSi (**Scheme 4**) to help better understand the interaction observed in this manuscript. However, the full understanding of the AHSi or soluble silica, and their biological effect is not fully understood and should be considered to be a fruitful endeavor for future study.

Scheme 4. H₂O₂, material property, and AHSi on dermal wound healing

4.5 Conclusions

10%wt silica nanoparticle-containing adhesive hydrogels were formulated to tune the H_2O_2 concentration released from the PEG-DA adhesive with various architecture. The robust design of the experiment was utilized to narrow down the candidate formulation and expected H_2O_2 concentration for dermal tissue repair application. FOX assays confirmed the amount of H_2O_2 can be estimated through robust design with a 10-20% deviation. Incorporation of silica particle effectively reduces the H_2O_2 concentration released from the adhesive regardless of their architecture. Lap shear tests confirmed the adhesive improve their adhesive strength with the addition of silica particles. The adhesive was successfully introduced in a full-thickness wound repair mice model. The result suggested that the adding catechol adhesive helped accelerate wound closure but fail to achieve a fully remodeled to healthy dermal tissue due to epidermal hyperplasia. The addition of AHSi proves to be beneficial for the adhesive in maintaining the ability to accelerate wound healing while reducing the epidermal hyperplasia. Ultimately, with a combination of appropriate adhesive architecture, H_2O_2 concentration, and addition of AHSi, an accelerated wound healing with better tissue remodeling was achieved. This proof of concept study strongly suggests the design of adhesive that considered the aforementioned factors to achieved tissue-specific response. However, a full understanding of the target tissue and their healing process is required to utilize this strategy effectively.

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5 Summary and Future Outlook

5.1 Summary

In Chapter 1, we established an understanding of ideal bioadhesive design and catechol based adhesive. The byproduct of catechol-based adhesive and the effect of H_2O_2 on biological response was discussed at length. We thus proposed the alternative approach to control the release of H_2O_2 from catechol adhesive with a biologically active silica particle incorporation.

Chapter 2 revolves around the detailed investigation of silica particle synthesis and the exploratory investigation on altering surface chemistry on the silica particle. In the later part of the chapter, we have describes the factor that directly affects silica particle synthesis, especially with Stöber synthesis approach. Several surface modification techniques have also been investigated with the resulting silica particle. This was further corroborated by various tests on the particle surface interaction with H_2O_2 in chapter 3 with various methods at physiological conditions.

Although the particle demonstrated promising characteristics and interaction with H_2O_2 , the application of such a particle is still unproven. In chapter 3, we investigate the effect of the particles on H_2O_2 generation and the effect of incorporating the particle on various types of cells and in the wound healing model in mice. We demonstrated that the particle still remains effective even after incorporation into PEG-DA adhesives in reducing H_2O_2 concentration. The particle also positively affects the biological response of RDF, HT,

and HEK, increasing cell proliferation and reduce toxicity in some formulation. The results were indicating that targeted-application and targeted-tissue adhesive design from such a concept can be achieved.

The ability of the silica particle and composite adhesives (reported in Chapters 2-3) to modulate the different concentrations of H_2O_2 was thought to be largely limited by the availability of surface binding sites as well as slow degradation of H_2O_2 . To address this concern, in Chapter 4, we study a wider range of adhesive formulations to find the extent of H_2O_2 modulation that the particle can provide. We demonstrated that, with careful selection of material and their control factors, a representative formulation from silica particle-PEG-DA composite could achieve the desired biological response in vivo via a full-thickness wound repair model in mice. The results strongly suggested that, with appropriate selection and control of H_2O_2 release level, dermal wound healing time can be reduced by at least 50% with a complete tissue remodeling.

5.2 Future Outlook

This dissertation describes the progress made toward controlling the H_2O_2 concentration during the oxidation of catechol adhesive through oxidative mediated oxidation by the introduction of surface activated silica particle for H_2O_2 sorption and complexation. The assays for multiple formulations of PEG-DA adhesives and particle composites were also included, which will facilitate the decision of formulation selection for future studies.

In Chapters 2-3, the particle was synthesized as a fully amorphous particle to allow better surface reactivity and ease of functionalization. Increasing the total surface area of the particle proves to be one of the effective modifications to increase the particle capability to react with H_2O_2 . Alternatively, post-synthesis heat treatments or adjustments during synthesis (i.e., increased temperature, change of silica precursor, etc.) can be made to alter the degree of crystallinity. A stronger functionalization method can still be used to alter surface chemistry, while increased crystallinity will likely increase the catalytic effect of the silica particle. Doping silica particles with metallic species might also result in a better catalytic degradation effect while maintaining the biocompatibility level of the composite as well. Since silica particle is easily modifiable, the possibility is nearly endless.

In Chapter 4, increasing or decreasing silica particle is also proved to drastically affect the biological response both in vivo and in vitro (as demonstrated in Chapter 3 and 4). However, the incorporation of the particle is seemingly more suitable for fine-tuning the H_2O_2 concentration range than utilizing solely to control the high amount of H_2O_2 . PEG-DA formulation proved to be one of the biggest deciding factors for the composite adhesive biological response as they are directly responsible for the H_2O_2 generation. Modifying the adhesive formulation and structure, then fine-tuning the adhesive bulk properties and H_2O_2 concentration level, could be a more effective approach to control the cytotoxicity and biological response. The author firmly believes that combining all the knowledge established in this dissertation would lead to a successful design of tissue-specific adhesive formulations with the ability to accelerate tissue repair.

6 References

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Appendix A.

NMR Spectra of PEG-D4

NMR Spectra of PEG-D6

NMR Spectra of PEG-D8

Prediction result from the robust design functions. The grey highlighted rows are the selected formulation.

Polymer type	Polymer conc.	PBS Conc.	Particle wt%	Gelation time	Max H2O2	Adhesive Strength
4	150	1	0	272.89	92.29	1.00
4	150	1	5	354.88	67.23	0.92
4	150	1	10	725.72	58.96	0.89
4	150	2	0	242.06	101.45	0.99
4	150	2	5	314.78	73.91	0.91
4	150	2	10	643.73	64.82	0.88
4	150	4	0	338.63	80.21	0.79
4	150	4	5	440.38	58.43	0.73
4	150	4	10	900.56	51.25	0.70
4	225	1	0	39.39	111.45	3.45
4	225	1	5	51.22	81.19	3.17

4	225	1	10	104.75	71.21	3.07
4	225	2	0	34.94	122.51	3.43
4	225	2	5	45.44	89.25	3.15
4	225	2	10	92.91	78.28	3.05
4	225	4	0	48.88	96.86	2.73
4	225	4	5	63.56	70.57	2.50
4	225	4	10	129.98	61.89	2.43
4	300	1	0	50.69	122.82	5.11
4	300	1	5	65.92	89.48	4.69
4	300	1	10	134.82	78.47	4.54
4	300	2	0	44.97	135.02	5.08
4	300	2	5	58.48	98.36	4.67
4	300	2	10	119.58	86.27	4.52
4	300	4	0	62.91	106.75	4.03
4	300	4	5	81.81	77.77	3.71

4	300	4	10	167.29	68.21	3.59
6	150	1	0	218.09	74.39	1.13
6	150	1	5	283.61	54.20	1.04
6	150	1	10	579.98	47.53	1.01
6	150	2	0	193.45	81.78	1.12
6	150	2	5	251.57	59.58	1.03
6	150	2	10	514.45	52.25	1.00
6	150	4	0	270.62	64.66	0.89
6	150	4	5	351.94	47.10	0.82
6	150	4	10	719.71	41.31	0.79
6	225	1	0	31.48	89.84	3.90
6	225	1	5	40.94	65.45	3.59
6	225	1	10	83.71	57.40	3.47
6	225	2	0	27.92	98.76	3.88
6	225	2	5	36.31	71.95	3.56

6	225	2	10	74.25	63.10	3.45
6	225	4	0	39.06	78.08	3.08
6	225	4	5	50.80	56.88	2.83
6	225	4	10	103.88	49.89	2.74
6	300	1	0	40.51	99.01	5.77
6	300	1	5	52.69	72.13	5.30
6	300	1	10	107.74	63.26	5.14
6	300	2	0	35.94	108.84	5.74
6	300	2	5	46.73	79.29	5.27
6	300	2	10	95.57	69.54	5.11
6	300	4	0	50.27	86.05	4.55
6	300	4	5	65.38	62.69	4.19
6	300	4	10	133.70	54.98	4.05
8	150	1	0	221.91	41.77	1.38
8	150	1	5	288.58	30.43	1.27

8	150	1	10	590.14	26.69	1.23
8	150	2	0	196.83	45.92	1.37
8	150	2	5	255.97	33.45	1.26
8	150	2	10	523.46	29.34	1.22
8	150	4	0	275.37	36.30	1.09
8	150	4	5	358.10	26.45	1.00
8	150	4	10	732.31	23.20	0.97
8	225	1	0	32.03	50.44	4.76
8	225	1	5	41.65	36.75	4.37
8	225	1	10	85.18	32.23	4.23
8	225	2	0	28.41	55.45	4.73
8	225	2	5	36.95	40.40	4.35
8	225	2	10	75.56	35.43	4.21
8	225	4	0	39.75	43.84	3.76
8	225	4	5	51.69	31.94	3.45

8	225	4	10	105.70	28.01	3.34
8	300	1	0	41.22	55.59	7.04
8	300	1	5	53.61	40.50	6.47
8	300	1	10	109.63	35.52	6.26
8	300	2	0	36.57	61.11	6.99
8	300	2	5	47.55	44.52	6.43
8	300	2	10	97.24	39.04	6.23
8	300	4	0	51.15	48.32	5.55
8	300	4	5	66.52	35.20	5.11
8	300	4	10	136.04	30.87	4.94